Márcia Figueiredo Gonçalves
Licenciatura em Bioquímica

Understanding how dendritic cell glycans affect antitumor immune responses

Dissertação para obtenção do Grau de Mestre em
Bioquímica para a Saúde

Orientador: Dr. Paula Alexandra Quintela Videira,
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"Cell surface Sialic acid removal from dendritic cells improves antigen cross-presentation and boosts anti-tumor immune responses" Mariana Silva*, Zélia Silva*, Graça Marques, Tiago Ferro, Mauro Monteiro, Márcia Gonçalves, Sandra Van Vliet, Elodie Mohr, Andreia Lino, Alexandra Fernandes, Yvette van Kooyk, Carlos Tadokoro and Paula A Videira

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Abstract

Dendritic cells (DCs) have a unique capacity to induce immune responses against tumor cells. They can phagocyte tumor antigens, maturate and present them to T cells, triggering antigen-specific immune responses that may lead to the elimination of tumor cells. Since they induce long-lasting immunological memory, DCs become an attractive strategy as cellular targets for vaccines in the treatment and/or prevention of cancer. However, the therapeutic results obtained in clinical trials with DCs are scarce and only few patients effectively respond to the DC vaccines. Our group has shown that sialic acid containing glycans play an important functional role in *ex vivo* generated DC. Here we aimed to establish an *in vitro* model to assess specific antitumor responses. To achieve this, an enzymatic treatment of monocyte-derived DCs (moDCs) was performed using sialidase to cleave surface sialic acids. The maturation profile of the moDCs was characterized by flow cytometry and cytokine expression. The results show that sialidase treatment can upregulate co-stimulatory molecules on surface of moDCs stimulated with Toll like receptor (TLR) agonists. To understand whether sialidase treatment affected the TLR signaling, we have used HEK cells stably transfected with TLRs 2, 4 and 7/8. The data showed that desialylation of moDCs does not affect the signaling via these receptors. To investigate the functional impact of sialidase treatment in the capacity of moDCs to present antigen and to activate antigen specific T cells, sialidase treated and untreated moDCs were co-cultured with $CD8^+$ T cell clones specific for peptides derived from the gp100 tumor antigen. Our results show that desialylated $HLA02:01^+$ DCs are superior in cross-presentation of the peptide to $gp100_{280–288}$ specific $CD8^+$ T cells. In addition, sialidase treatment also increased the DC capacity to induce $CD4^+$ T cells proliferation. Together, these data indicate that moDCs with altered cell surface sialic acids, through a sialidase treatment, have a better immunostimulatory potential which could improve anti-tumor immune responses.

Key words: moDCs, glycans, sialidase, desialylation, therapy, gp100 peptide, TLR

Resumo

As células dendríticas (DCs) têm a capacidade única de induzir respostas imunitárias contra as células tumorais, fagocitando antigénios tumorais e apresentando-os às células T, provocando respostas imunitárias específicas que conduzem à eliminação de células de tumorais. Por induzirem memória imunológica de longa duração, as DCs são uma estratégia atrativa para o tratamento e/ou prevenção do cancro. No entanto, os resultados terapêuticos obtidos em ensaios clínicos com DCs são escassos e pouco eficientes. O nosso grupo demonstrou que ácidos siálicos que contêm glicanos desempenham um papel funcional importante em DCs geradas *ex vivo*. Com o objetivo de estabelecer um modelo *in vitro* para avaliar a resposta anti-tumoral específica realizou-se um tratamento enzimático a DCs derivadas de monócitos (moDCs) com sialidase, enzima que cliva ácidos siálicos na superfície celular. O perfil de maturação de moDCs foi caracterizado por citometria de fluxo e expressão de citocinas. Os resultados mostram que a sialidase pode regular positivamente a expressão de moléculas co-estimuladoras na superfície de moDCs estimuladas com agonistas de *Toll like receptors* (TLRs). Para percebermos se o tratamento com sialidase afeta a sinalização dos TLRs foram usadas células HEK transfectadas de forma estável com TLRs 2, 4 and 7/8. Os dados mostraram que a desialilação não afeta a sinalização através estes recetores. Para investigar o impacto funcional da sialidase na capacidade de moDCs em apresentar um antigénio e ativar células T, moDCs foram tratadas, ou não, com sialidase e cultivadas com clones de células T $CD8^+$ específicas para os péptidos derivados do antigénio tumoral gp100. Os resultados mostram que DCs $HLA*02:01^+$ desialiladas exibem maior *cross-presentation* do péptido $gp100_{280-288}$ às células T $CD8^+$ específicas. Além disso o tratamento com sialidase também aumenta a capacidade de DCs de induzir a proliferação de células T $CD4^+$. Em conjunto, os resultados indicam que moDCs com menos ácidos siálicos na superfície, têm melhor potencial imuno-estimulador, com maior capacidade de induzir respostas imunes anti-tumorais.

Palavras-chave: moDCs, glicanos, sialidase, desialylation, terapia, peptídeo gp100

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Abbreviations

Ag – Antigen

AMV RT - Avian Myeloblastosis Virus Reverse Transcriptase

APCs – Antigen Presentation Cells

BCR – B Cell Receptor

BSA – Bovine Serum Albumin

CLR – C-type Lectin Receptor

CTLs – Cytotoxic T Lymphocytes

DCs – Dendritic Cells

DC-SIGN - DC-specific ICAM-3 grabbing non-integrin

dNTP - Deoxyribonucleotides Phosphated

ELISA - Enzyme-Linked Immunosorbent Assay

ER – endoplasmic reticulum

FDA - Food and Drug Administration

FITC - Fluorescein isothiocianate

GalNAc - N-acetylgalactosamine

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GM-CSF - Granulocyte macrophage colony-stimulating factor

GMO – Genetically modified

HBSS - Hank’s buffered saline solution

HEK – Human Embryonic kidney

HLA – Human Leukocyte Antigen

HPV – Human Papillomavirus

HSCs – Hematopoietic Stem Cells

IFN-γ – Interferon-gamma

IL – Interleukin

Io – Iomycin

LCs – Langerhans cells

LPS – Lipopolysaccharide

MAA II - *Maackia amurensis* agglutinin (*O*-glycan)

MACS - Magnetic-activated cell sorting

MAL I - *Maackia amurensis* lectin (*N*-glycan)

mDC – Myeloid Dendritic Cell

MFI – Mean Fluorescence Intensity

MHC – Major Histocompatibility Complex

moDCs – monocyte-derived dendritic cells

Neu5A - N-acetylneuraminic acid

NF-κB - Nuclear factor kappa β

NK – Natural Killer cell

NOD – nucleotide-binding oligomerization domain receptors

ON – overnight

PAMP – Pathogen Associated Molecular Patterns

PBA - PBS/0.5% BSA

PBLs – Peripheral blood lymphocytes

PBMCs – Peripheral blood mononuclear cells

PBS – Phosphate buffered saline solution

pDC – Plasmacytoid Dendritic Cell

PE – Phycoerythrin

PMA – Phorbol Myristate acetate

PRRs – Pattern Recognition Receptors

qPCR - quantitative polymerase chain reaction

RT – Room Temperature

Siglecs - Sialic acid-binding Ig-like lectins

SNA - *Sambucus nigra* agglutinin

TAP – Transporter for Antigen Presentation

TCR – T Cell Receptor

Th – helper T lymphocyte

TLR – Toll-like Receptor

TMB - 3,3′,5,5′-tetramethylbenzidine

TNF-α – Tumoral Necrosis Factor – α

Treg – regulatory T cell

Dissertation

1) Introduction

1.1) Cancer

For a good functioning of the human body a balance must exist between proliferation and cell death (Baehrecke, 2002). However, due to external or internal conditions, mutations occur during the generation of new cells which could lead to disruption of this homeostasis since these cell lose the ability to respond to stimulus (Kumar, V., Abbas A. K., Fausto, N. & Mitchell, 2007).

Transformation of normal cells into cancer cells is associated with phenotypic changes that affect the behavior of cells and consequently other biological processes. Hanahan and Weinberg (Hanahan e Weinberg, 2011), described the main aspects that characterize cancer: evasion of apoptosis, inadequate signs to inhibit cell growth, the ability to proliferate indefinitely, acquiring modifications that allow invasion of other tissues and tumor vascularization. All of them allow tumor cell survival and uncontrolled cell proliferation that leads to invasion of other tissues and subsequent metastasis (Bogenrieder e Herlyn, 2003; Goldsby, R.A., Kindt, T. J., Osborne, B., Kuby, 2003; Hanahan e Weinberg, 2011; Kumar, V., Abbas A. K., Fausto, N. & Mitchell, 2007), the main cause of death in people with cancer (EL, 1997).

These characteristics (Hanahan e Weinberg, 2011) distinguish cancer cells from normal cells, potentially allowing the tumors to be recognized as "foreign" by the immune system (Chen, Irving e Hodi, 2012; Gros *et al.*, 2014; Mellman, Coukos e Dranoff, 2011; Rooij, van *et al.*, 2013). However, tumors are rarely rejected spontaneously, reflecting their ability to maintain an immunosuppressive microenvironment (Chen e Mellman, 2013). Thus, it is necessary around this to capture tumor antigens by antigen presenting cells (APCs) (such as dendritic cells) so that these tumor epitopes are processed and presented to immune effector cells (lymphocytes) (Arosa, A. F., Cardoso, E.M., 2011).

In 2012, worldwide (World cancer research fund international) 14.1 million new cancer cases were diagnosed of which 8.2 million died. It is estimated that in 2035 the number of persons with cancer will increase dramatically to 24 million.

Conventional therapies such as surgical resection and a combination of chemotherapy and radiotherapy are approaches that are often accompanied by unintended collateral damage and with high toxicity to healthy tissues and have not been sufficiently effective in the fight against cancer. It is of great importance to develop a therapy that uses the patient's own immune system and, in that way, ensures the safety of patients while being effective in eliminating the tumor.

1.2) Immune system

The immune system has the ability to respond to different external aggressions, in particular antigenic natures that are foreign to the body, whether a microorganism or macromolecule, and various internal injuries, or modified tumor cells. Thus, you can define immunity as a set of defense mechanisms that our body has to protect itself from attacks, to maintain the immune homeostasis. The immune system under normal conditions doesn´t present any kind of response to host cells, a situation that it is known as immunological tolerance (Arosa, A. F., Cardoso, E.M., 2011).

1.2.1) Innate and adaptive immunity

The reaction of the immune system can be divided into two types of interconnected pathways: innate immunity and adaptive immunity.

Innate immunity is the first line of the body's defense and it is mainly mediated by neutrophils, macrophages, natural killer cells (NK) and dendritic cells (DCs), commonly

known as phagocytic cells, which phagocytose the foreign body and destroy it (Akira, 2011).

This is an immediate response with a broad spectrum specificity, recognizing only molecular patterns stored in microorganisms or PAMPs (Pathogen-associated molecular patterns) through specific receptors on phagocytic cells (macrophages and DCs). These receptors are called pattern recognition receptors (PRRs) and include the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), such as the mannose receptor. TLRs are able to recognize a wide variety of PAMPs. This recognition results in the activation of phagocytic cells that can lead to the internalization of the microorganism, as well as the release of cytokines and inflammatory mediators, triggering an inflammatory process (Arosa, A. F., Cardoso, E.M., 2011). In contrast to adaptive immunity, each time the body is exposed to the pathogen, the immune response is always the same, since there is no memory on previous exposures.

Cytokines released by cells from inflamed tissues, such as interleukin-1 (IL-1) and interleukin-6 (IL-6) are important in activation of immune response and cell interaction. Then, mononuclear cells and lymphocytes, that in the meantime, are attracted to the inflammatory focus, are activated and start releasing their own cytokines (IL-1, IL-2, IL-4, TNF-α, IFN- γ, etc.) enhancing and promoting the migration and activation of certain cells more directly involved in the immune response (Akira, 2011; Arosa, A. F., Cardoso, E.M., 2011).

Adaptive immunity is the more delayed response of the immune system and requires the clonal proliferation of effector cells (T and B lymphocytes), which have to capacity to form memory cells and is therefore more effective (Seeley, 2003). This response requires activation of T and B lymphocytes, which is mainly performed by professional APCs, such as DCs, that after the capture of antigen, processes it and migrate to the lymph nodes where it is presented to T cells (fig.1).

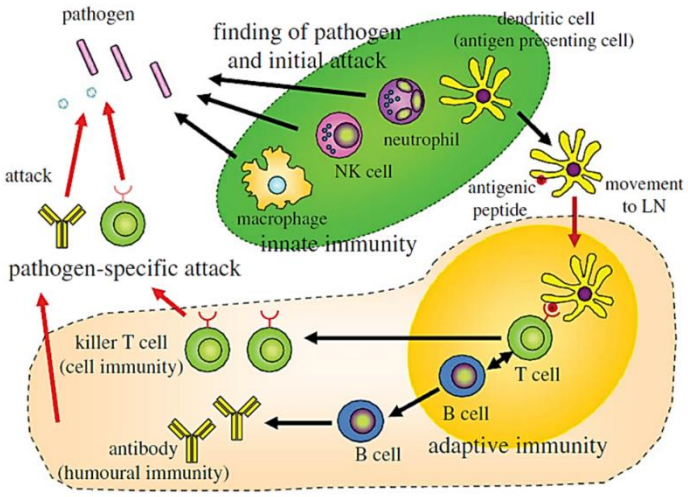


Figure 1 - Immune System: Innate immunity and adaptive immunity.
Addapted from Akira et al., (Akira, 2011).

While the B cell receptor recognizes the antigen in its native form (not processed), the T cell receptor only recognizes the antigen in a fragmented form (for example peptides) presented in the context of MHC (Major Histocompatibility Complex) molecules. This recognition results in activation and differentiation of B lymphocytes, which will produce antibodies specific to the antigen, and the activation and differentiation of T lymphocytes, which lead to the formation of effector cells and regulatory cells of the immune response (Arosa, A. F., Cardoso, E.M., 2011).

1.2.2) T lymphocytes

T cells are a lymphocyte lineage originated from bone marrow hematopoietic precursors which complete their maturation in the thymus. These cells are roughly divided into four groups: helper T lymphocytes (Th), cytotoxic T cells (CTL), regulatory T cells (T_{reg}) and natural killer T lymphocytes (NKT) (Arosa, A. F., Cardoso, E.M., 2011).

The Th cells express CD4 and their function is to produce cytokines that, in turn, will help in the activation of other immune cells. The formation of the tri-molecular complex TCR/peptide /MHC allows the presentation of the peptide by APCs to Th cells,

which is restricted by antigen presentation in MHC class II molecules. After activation, the Th lymphocytes begin a differentiation process, and can develop into four types of lymphocytes: Th1, Th2 and Th17 (Goldsby, R.A., Kindt, T. J., Osborne, B., Kuby, J, 2007).

The Th1 lymphocytes are characterized by production of IL-2, IFN-γ and TNF-β. The function of IL-2 is to regulate the growth of Th1 cells as well as $CD8^+$ T lymphocytes.

The Th2 lymphocytes are characterized by production of IL-4, IL-5, IL-9, IL-10 and IL-13 (anti-)inflammatory cytokines responsible for regulating the humoral response against extracellular pathogens and allergens (Goldsby, R.A., Kindt, T. J., Osborne, B., Kuby, J, 2007).

The CTL express CD8, and their function is to eliminate other cells, particularly when they have become a tumor or are infected. The CTL response is restricted by antigen presentation in MHC class I molecules and is characterized by production of the cytokines IFN-γ and TNF-α and lytic enzymes (perforin and granzymes), responsible for the elimination of target cells (Arosa, A. F., Cardoso, E.M., 2011).

The T_{reg} derived from a separate "lineage" of lymphocytes (not Th) and function as controllers of the immune response and play an important role in the maintenance of immunological tolerance to innocuous antigens in the periphery and in the prevention of autoimmunity, allergies and, in general, are responsible for the suppression of immune responses (Lehtimäki e Lahesmaa, 2013).

The NKT cells contain mixed features of T and NK lymphocytes and are determinants of infectious and autoimmune diseases. NKT cells express a particular type of TCR that recognizes glycolipid antigens in the context of non-classical MHC CD1d and once activated are cytotoxic and produce cytokines such as IL-4 and IFN-γ (Wu e Kaer, Van, 2009). Although NKT can directly eliminate tumor cells, it is believed that their effectiveness arises from the effect on NK cells, $CD8^+$ T cells and DCs, leading, particularly in DCs, to their activation/maturation via IFN-γ production (Terabe e Berzofsky, 2008).

T cell activation begins with the recognition by the TCR/CD3 of antigenic peptides exposed on the surface of APCs in association with MHC molecules. The signal from the TCR /CD3-MHC-peptide interaction is the primary activation signal ("signal 1"), which confers specificity to the adaptive response. However, this signal in itself is not effective to induce strong activation that causes the naive $CD4^+$ T and naive $CD8^+$ T lymphocytes to enter the cell cycle and to proliferate, making it necessary to receive accessory signals. These accessories signals are transmitted by the CD28 receptor, considered "Signal 2", which will lead to the formation of $CD4^+$ T and $CD8^+$ T lymphocytes with different phenotypic and functional characteristics than the initial naive T lymphocyte (Arosa, A. F., Cardoso, E.M., 2011).

1.3) Dendritic cells

1.3.1) Origin, differentiation and classification

DCs were identified for the first time by Paul Langerhans in 1968, who mistakenly thought they were part of the nervous system (Langerhans cells, LCs) (Goldsby, R.A., Kindt, T. J., Osborne, B., Kuby, J, 2007). This concept held until the mid-twentieth century. In 1973 Ralph Steinman and Zanvil Cohn observed a cell population in the spleen with a dendritic shape and demonstrated that this was a new class of cells with immunomodulatory functions in the immune system (Paczesny S., 2003).

Currently, it is known that DCs originate from $CD34^+$ hematopoietic stem cells (HSCs) from the bone marrow. In physiological stress conditions, monocytes can differentiate into immature DCs in the presence of the stimulating factor, granulocyte macrophage colony (GM-CSF) and from a variety of other cytokines (Mogensen, 2009).

The enormous heterogeneity presented by DCs makes for its rather complex classification, however, generally it can be classified as plasmacytoid DCs (pDCs), or myeloid DCs (mDCs) depending on specific characteristics, such as the expression of

surface markers, location in the body. Both mDCs and pDCs perform specific functions and specific inflammatory stimulus that induce differentiation (Arosa, A. F., Cardoso, E.M., 2011).

The pDCs can be derived from myeloid precursor and are in the blood and lymphoid organs (Sathe *et al.*, 2013). These cells are characterized by their extraordinary ability to produce interferon type 1 (IFN-α / β) following viral infection or after interaction with TLRs 7 and 9 agonists. From a functional standpoint, pDCs have an enormous plasticity and can induce Th responses (Th1 and Th2), or tolerance via induction of T_{reg} cells. Moreover, they are still capable to perform antigen cross-presentation via MHC class I, due to their special endosomal compartments (Arosa, A. F., Cardoso, E.M., 2011; McKenna, Beignon e Bhardwaj, 2005).

Likewise, mDCs are from the myeloid lineage and can be found in tissues (Langerhans cells and interstitial DCs) and in peripheral blood (inflammatory DCs) (Brussel I.V., Berneman Z.N., 2012; Paczesny S., 2003; Zhang, C. e Engleman, 2006). Inflammatory DC are involved in recognition of bacterial structures and production of pro-inflammatory cytokines, including tumor necrosis factor α (TNF-α), IL-6 and IL-12p70 to activate Th1 / Th17 cells, and thus recruiting CTLs (Arosa, A. F., Cardoso, E.M., 2011; Zhang, C. e Engleman, 2006).

It has been further shown that pDCs can enhance the immune response through cross-talk with mDCs through IFN-γ production and CD40L expression, enabling the production of IL-12p70 by the mDC (Boudreau J. E., Bonehill A., Thielemans K., 2011).

1.3.2) Antigen recognition and uptake

Acting as sentries in peripheral tissues, the main function of DCs is antigen presentation to T lymphocytes. These cells are considered to be professional APCs and are able to endocytose and process any type of antigen and to present it in the context of MHC molecules. For this reason the DCs are better equipped compared to macrophages and B cells. Another feature that distinguish them as professional APCs, is the so-called

maturation process in which immature DC with large endocytic capacity and low expression of MHC-II molecules, differentiate into mature DC characterized by a low endocytosis capacity and a high expression of MHC-II molecules. The immature DCs, lying in organs and peripheral tissues, have a great ability to endocytose and process endogenous and exogenous antigens while mature DCs are specialized in the activation of T lymphocytes in secondary lymphoid organs.

Generally, endogenous antigens presented by DCs in the context of MHC-I activate $CD8^+$ T lymphocytes, while exogenous antigens are presented by DCs in the context of MHC II activate $CD4^+$ T lymphocytes. DCs also have the unique ability to present exogenous antigens *in vivo* to $CD8^+$ T lymphocytes via MHC-I, in a process called cross-presentation (Arosa, A. F., Cardoso, E.M., 2011; J. E. Boudreau, A. Bonehill, K. Thielemans, 2011).

1.3.2.1) Receptors in the antigen recognition and uptake

The designated PAMPs, which are highly conserved structures including microbial lipids, polysaccharides, nucleic acids and viral RNA, are recognized by immature DCs through PRRs. These receptors are highly diverse, which includes TLRs, nucleotide-binding oligomerization domain (NOD-like) receptors and CLRs (Kanazawa, 2007; Mogensen, 2009).

After antigen recognition, their internalization is mediated by a large number of endocytic and phagocytic receptors including CLRs and integrins.

The TLRs are transmembrane proteins which are present in DCs, macrophages, fibroblasts and epithelial cells. They are involved in recognition of antigens and subsequent activation of cell signaling pathways. mDCs express TLR 1–8, which when stimulated, upregulate activation markers (CD80, CD86, MHC class I and II), produce pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-12), chemokines, adhesion molecules (ICAM-1) and prime antigen-specific $CD4^+$ and $CD8^+$ T cells (Gnjatic, Sawhney and Bhardwaj, 2010). Activation of TLRs by exogenous or endogenous ligands induces DC

maturation and activation, thereby determining the onset of immune responses (Arosa, A. F., Cardoso, E.M., 2011).

Table 1 - Synthetic ligands of Toll-like receptors and respective PAMPs.
Adapted from Gnjatic et al.,(Gnjatic, Sawhney and Bhardwaj, 2010).

Receptor	**Pathogen Associated ligands (PAMPs)**	**Synthetic ligands**
TLR ½	Triacylated lipopeptides (Bacteria and Mycobacteria)*	Pam3CysK4
TLR 4	Lipopolysaccharide (LPS) (Gram-negative bacteria);	LPS
TLR 7	Viral ssRNA (Influenza, VSV, HIV, HCV)	Guanosine analogs; imidazoquinolines (e.g.R848, Resiquimod®); Loxoribine.
TLR 8	ssRNA from RNA virus	Imidazoquinolines (e.g.R848, Resiquimod®); Loxoribine.

NOD-like receptors are located in cytoplasm of the DC and are capable of binding to bacterial peptidoglycans (Kanazawa, 2007).

The CLRs recognize and internalize antigen to the intracellular compartments of DCs, which subsequently leads to processing and presentation of the antigens via MHC-I and MHC-II. The mannose receptor/CD206, langerin/CD207, present on Langerhans cells, DC-SIGN/CD209 and DEC205/CD205 are examples of CLRs (Kanazawa, 2007). Recent studies have demonstrated that CLRs are involved in antigen internalization by DCs, in activation of intracellular signaling pathways including nuclear transcription factor kappa B (NF-kB), and also in inducing cytokine expression which determines the polarization of Th lymphocytes (Arosa, A. F., Cardoso, E.M., 2011).

1.3.3) Antigen processing

Before antigen detection, DCs are in an immature stage. After recognition, DCs phagocytose the antigen and then enter an activation process, maturation, and migrate to the lymph nodes where a T cell immune response specific for the antigen is initiated. During the process of maturation and migration, DCs process the antigen into smaller fragments that may be presented to T lymphocytes (Hamdy *et al.*, 2011).

DCs process endogenous and exogenous antigens, presenting them to T lymphocytes in the form of antigenic peptides bound to MHC molecules (Hamdy *et al.*, 2011). This processing is different based on the origin and the molecular nature of antigen, whereby presentation occurs via three mechanisms: i) via MHC class I or cytosolic (endogenous); ii) via MHC class II or endocytic (exogenous); iii) presentation of lipid antigens coupled to CD1 molecules (Goldsby, R.A., Kindt, T. J., Osborne, B., Kuby, J, 2007).

Extracellular antigens are captured by endocytosis, phagocytosis and pinocytosis, which enter the endocytic pathway, forming endosomes that subsequently undergo maturation and fusion with lysosomes. In lysosomes hydrolytic enzymes cleave the antigen into smaller molecules, peptides, which are then coupled to MHC-II molecules. The MHC-peptide complex is transported to the cell surface during the process of dendritic cell maturation for antigen presentation to $CD4^+$ T naive lymphocytes (Goldsby, R.A., Kindt, T. J., Osborne, B., Kuby, J, 2007).

Through the cytosolic pathway, intracellular antigens (which may be self-proteins or of pathogenic or viral origin) are ubiquitinated and degraded by proteasomes into peptides. Through the transporter associated with antigen processing (TAP) the peptides are directed to the endoplasmic reticulum where they bind to MHC-I molecules. The MHC I-peptide complex is then transported to the cell surface where antigen presentation to naive $CD8^+$ T cells can occur (Goldsby, R.A., Kindt, T. J., Osborne, B., Kuby, J, 2007).

1.3.4) DC maturation

In peripheral tissues DCs are usually in an immature state and practically devoid of immunostimulatory activity. However, after a stimulus (danger signal - antigen) they acquire different morphological, phenotypic and functional properties. In an immature state, DCs have a low ability to stimulate immune responses and a high power to capture antigens. When the maturation process starts, DCs lose their phagocytic receptors and increase their migration from peripheral tissues to secondary lymphoid organs where they present antigen to naive lymphocytes, culminating in the acquisition of immunostimulatory potential (maturation). antigen processing is regulated in a coordinated manner through maturation of the DC, which causes a decrease in pH of the endosomes, whereby processing is facilitated, enabling the transport of the MHC-peptide complex to the cell surface (Arosa, A. F., Cardoso, E.M., 2011).

Indeed, mature DCs express high levels of costimulatory molecules, as well as MHC molecules and synthesize high levels of IL-12 which enhances the ability to induce innate (NK cells) and adaptive (T and B cells) responses (Brussel I.V., Berneman Z.N., 2012). This process is continuous and highly regulated by signal transduction pathways that can be triggered directly through recognition of pathogens via PRRs or indirectly by exposure to other inflammatory mediators produced by immune cells, which results in increased membrane expression of costimulatory molecules (Brussel I.V., Berneman Z.N., 2012; Neves, 2010). The increased expression of MHC class I and class II and costimulatory molecules CD40, CD83, CD80 and CD86 during maturation is crucial in order to establish the immunological synapse and consequent stimulation of lymphocytes (Arosa, A. F., Cardoso, E.M., 2011).

The stimuli that induce maturation include products of microorganisms that bind to pattern recognition receptors, immune complexes that act on Fc receptors, inflammatory molecules released from the host cells, particularly CD40L, TNF-α, IL-1β, IL-6 e IFN-γ, and molecules released from damaged tissues such as uric acid (Arosa, A. F., Cardoso, E.M., 2011; Wu, Wang e Zhang, 2004).

1.3.5) Antigen presentation by DCs to T cells

In the lymph nodes, DCs present antigen to $CD4^+$ and $CD8^+$ T cells via MHC II and MHC-I, respectively. This interaction results in the activation of $CD8^+$ T lymphocytes and the differentiation of $CD4^+$ T lymphocytes in their different effector and/or regulatory cells. For this it is necessary that the antigenic recognition via MHC and interaction between costimulatory molecules on DCs and their respective ligands on T cells occurs (J. E. Boudreau, A. Bonehill, K. Thielemans, 2011).

In addition, DCs also have a unique ability to present exogenous antigens via MHC-I molecules. This function is referred to as cross-presentation of antigens. Exogenous antigens can be derived from apoptotic tumor cells or apoptotic infected cells (viral or bacterial). These antigens are degraded by the proteasome and coupled to MHC-I molecules for presentation to naive $CD8^+$ T lymphocytes. This process ensures that DCs are able to create a cytotoxic immune response against tumor or infected cells. However, the cross-presentation process can yield, by the $CD8^+$ T cells both effective immunity (cross-priming) and tolerance (cross-tolerance). Nevertheless, this mechanism has been crucial in the formulation of anti-tumor vaccines (Arosa, A. F., Cardoso, E.M., 2011; Goldsby, R.A., Kindt, T. J., Osborne, B., Kuby, J, 2007; Hamdy *et al.*, 2011).

The lipid antigens present in microorganisms (mycobacteria) or endogenous tissues, are presented to the lymphocytes by DCs through CD1 molecules. These are essential in presenting specific glycolipids to NKT cells. Plasmacytoid DC do not have CD1 molecules (Arosa, A. F., Cardoso, E.M., 2011; Goldsby, R.A., Kindt, T. J., Osborne, B., Kuby, J, 2007).

Moreover, the role of DCs in the activation of B cells is mainly indirect, through induction of expression of CD40L and IL-2 on T lymphocytes (important factors in the activation of B lymphocytes).

In conclusion, DCs are central in the initiation and regulation of adaptive immune responses which makes these cells a promising target in anti-tumor treatments (Benencia *et al.*, 2012).

1.4) Anti-tumor immunity

Activating the immune system for therapeutic benefit in cancer has long been a goal in immunology and oncology.

The generation of an effective anti-tumor immune response is a complex multistep process and the understanding of this matter provides a rationale for immunotherapeutic strategies (Buckanovich *et al.*, 2008; Mellman, Coukos e Dranoff, 2011; Pardoll, 2012). Advances in the understanding of how tolerance, immunity and immunosuppression regulate anti-tumor immune responses suggest that active immunotherapy represents a means to obtain a durable and long-lasting response in cancer patients.

For anti-tumor immunity to be effective three distinct steps must be achieved, either spontaneously or therapeutically: immunization, T cell response and blocking immunosuppression (fig.2) (Mellman, Coukos e Dranoff, 2011).

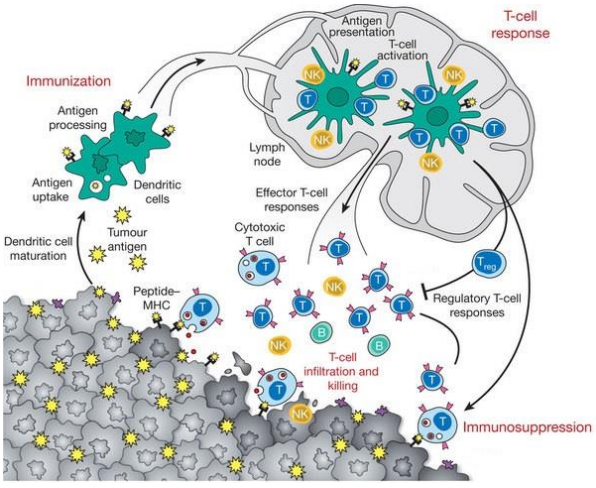


Figure 2 – Sites for therapeutic intervention that allow regulated anti-tumoral immunity. Increased presentation of tumor antigens by mature dendritic cells leads to more effective adaptive immune response mediated by T cells and help to prevent immunosuppression. Adapted from Mellman et al., (Mellman, Coukos e Dranoff, 2011).

The anti-tumor response must begin with the uptake of tumor-associated antigens, exogenously or captured from dead or dying tumor cells by immature dendritic cells.

The dendritic cells process the captured antigen for presentation or cross-presentation on MHC class II and class I molecules, respectively, and migrate to draining lymph nodes. However, during capture and presentation it is necessary an adequate stimulus (activation signal) to mature dendritic cells is present. This stimulus depends of the concentration of the antigen, and the intensity and duration of the interaction with lymphocytes.

Activation signals could be therapeutically supplied in an exogenous (for example, TLR - ligands) or endogenous manner: dying or necrotic tumor cells release factors (for example, high mobility group proteins or ATP) that are thought to result in the immunogenic maturation of dendritic cells (Mellman, Coukos e Dranoff, 2011). Thus it initiated the antitumor response mediated by anti-cancer effector T cells.

The T cells of the immune system must first be able to recognize cancer cells as foreign, to generate a population of CTLs that can traffic to and infiltrate tumors wherever they reside, and specifically bind to and kill cancer cells (Chen, Irving e Hodi, 2012). However, if there is no accompanying maturation, DCs can induce tolerance leading to the elimination of T cells, T cell anergy or differentiation of T_{reg} cells (Chen, Irving e Hodi, 2012).

In addition to the activation of $CD8^+$ and $CD4^+$ T cells, dendritic cells may also trigger antibody and NK or NKT cells responses, which may contribute to the tumor immunity (Mellman, Coukos e Dranoff, 2011)

Additionally, tumors may downregulate their expression of MHC class I molecules or their expression of target tumors antigens (Hamanishi *et al.*, 2007; Kooi *et al.*, 1996). The number of predicted MHC Class I-associated neoantigens was correlated with cytolytic activity and was lower than expected in colorectal and other tumors, suggesting immune-mediated elimination (Rooney *et al.*, 2015).

Therefore, the importance of the maturation state of DCs is essential and key to whether the type of anti-tumor response is adequate and effective.

1.5) Immunotherapy

The conventional treatment for cancer includes routinely surgical resection and a combination of chemotherapy and radiotherapy. These approaches are often accompanied by unintended collateral damage and highly toxic to healthy tissues, which are offset by only marginal improvements in prognosis in patients with advanced cancers. This unfortunate balance has driven the development of new therapies aimed at achieving tumor elimination both safely and efficiently.

Over the last decade, increasing evidence supports a therapeutic utility of the immune system by immunotherapy. The aim of this strategy is to enable, restore, manage and still complement the patient's own immune system to control tumor growth and dissemination (Aris e Barrio, 2015). Sometimes pre-existing anti-tumor T cells can be ineffective in the elimination of the tumor for several reasons: due to their low frequency, the selection of tumor cells to escape recognition by the immune system, or even because these lymphocytes are functionally disabled (Aris e Barrio, 2015).

Among other immunotherapy approaches, vaccines can be prophylactic and therapeutic (Palucka, Banchereau e Mellman, 2010). Prophylactic (or preventative) vaccines have been used with considerable success for the prevention of cancers of viral origin, such as hepatitis B virus and human papillomavirus (HPV), where the etiological agent is known. In contrast, the development of therapeutic vaccines to treat existing disease has proven problematic. The long history of failure has tainted the entire strategy of immunotherapy in the eyes of many oncologists.

The idea of a therapeutic cancer vaccine began with the discovery that patients can harbour $CD8^+$ and $CD4^+$ T cells specific for cancer-testis or differentiation antigens expressed in their tumors (Boon *et al.*, 2006). Vaccination might reasonably be expected to

amplify the frequency and strength of these pre-existing responses or perhaps induce some *de novo* reactions. Therapeutic vaccines can be administered as adjuvant therapy after excision of tumors, with the aim of overcoming immunosuppression produced by the tumor and its microenvironment, to stimulate specific immune effectors that can destroy tumor cells or to increase the immunogenicity of the tumor to induce long-lasting immunity.

Additionally, clinic-pathological studies have demonstrated a strong association between prolonged patient survival and the presence of intra-tumoral $CD8^+$ cytotoxic T cells and an IFN-γ gene signature (Galon *et al.*, 2006; Zhang *et al.*, 2003). Thus, if vaccination could trigger these types of T cell responses, a clinical benefit might be expected.

In general, vaccines require two critical components, the source of Antigen and adjuvant (Batchu *et al.*, 2005). Therapeutic vaccines include the use of different antigenic sources, such as, antigen peptides, proteins, nucleic acids, tumor cells lysates, recombinant virus or irradiated whole cells (Aris e Barrio, 2015).

1.6) Vaccines based on DCs

Alternatively, since the mid-90s, DCs have been used in clinical trials as cellular mediators for therapeutic vaccination of cancer patients (Anguille *et al.*, 2014). The use of DCs in an immunotherapeutic strategy is based on its ability to initiate cellular immune responses through the stimulation of naive T cells. Immature DCs are good at antigen uptake and processing, but for a stimulatory T-cell response they must mature to become fully activated DCs, which express high levels of cell surface-related MHC antigen and costimulatory molecules. Because of their ability to stimulate T cells, DCs act as a link between innate immunity and adaptive immunity in anti-tumor immune responses (Banchereau e Steinman, 1998).

DCs play a central role in various immunotherapy protocols aimed at the generation of CTLs (Reid, 2001). DC-based vaccines have become the most attractive tool for cancer

immunotherapy and have been used in the treatment of more than 20 malignancies, most commonly melanoma, renal cell carcinoma, prostate cancer, and colorectal carcinoma (Palucka, Ueno e Banchereau, 2011; Ridgway, 2003). Since tumor antigen-loaded DCs are expected to be able to stimulate tumor-specific CTLs and to overcome T cell tolerance in tumor patients, the development of DC vaccines that can consistently eliminate minimal residual neoplastic disease remains an important goal in the field of tumor immunology (Banchereau e Palucka, 2005).

However, much skepticism has been shown due to the uncertainty of their clinical efficacy, since only some patients have an effective response. However, the clinical benefit of DC-based immunotherapy is small, but real, whereby 8.5% of patients with melanoma achieved an objective response. The DC therapy is as effective as dacarbazine, the standard of care chemotherapy, where 5-15% of patients show an objective response (Anguille *et al.*, 2014).

In 2010, the FDA approved Sipuleucel-T, the first DC-based vaccine for the treatment of metastatic castrate resistant, hormone refractory prostate cancer (Beer *et al.*, 2011).

1.6.1) DC vaccine optimization

The key step in this approach lies in producing a DC vaccine capable of eliciting an immune response that is capable of destroying the tumor. The vaccines based on DCs are composed of DCs that are generated from peripheral blood precursors (i.e., monocytes, HSCs) or bone marrow progenitor cells and are educated *ex vivo* with tumor antigens prior to vaccination in patients (fig.3) (Lee *et al.*, 2012; Steinman e Banchereau, 2007) .

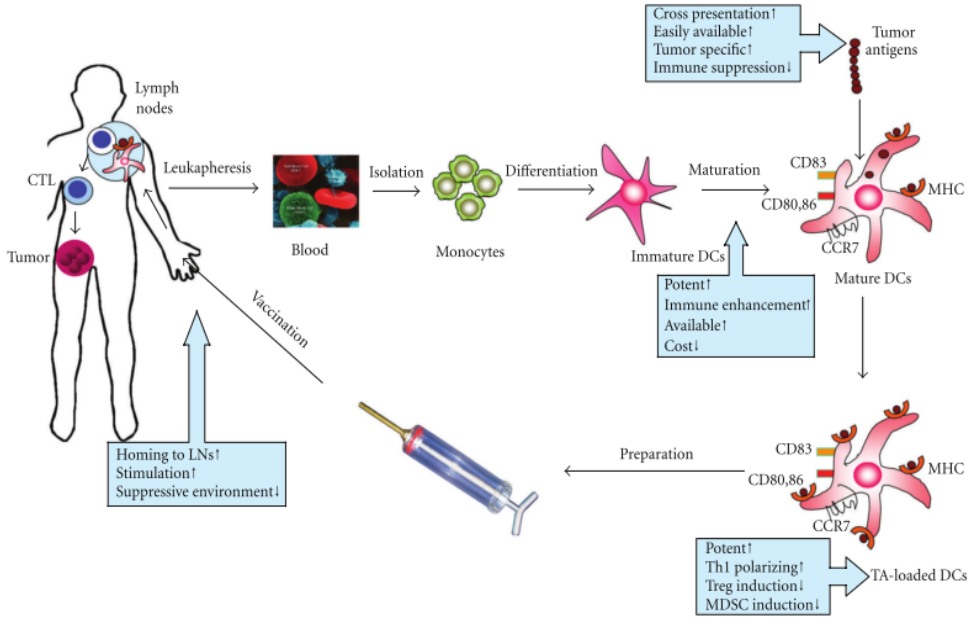


Figure 3 - Key points to improve DC vaccination in cancer patients. Adpated from Lee et al., (Lee *et al.*, 2012)**.**

Despite this simplistic picture (fig.3), there are several strategies used to activate DCs and to obtain a more effective vaccine. The use of immature DCs or mature DCs, the way to induce DC maturation, the type of tumor antigen, the techniques used to load tumor antigens to DCs, routes of administration, and dosing schedules are all being investigated (Figdor *et al.*, 2004).

It has been demonstrated by several authors (Boog *et al.*, 1989; Cabral *et al.*, 2013; Crespo *et al.*, 2009; Jenner *et al.*, 2006) that changes in the glycosylation of the dendritic cell surface, more specifically sialylation, influences the subsequent activation of these cells and their role in induction of immune responses.

1.7) Glycosylation

Glycosylation is a post-translational modification more commune in proteins of eukaryotic cells has a frequency of 50% (Hang e Bertozzi, 2005; Wopereis *et al.*, 2006). This process involves covalent attachment of one or more glycans consisting of

monosaccharides to a protein, lipid, carbohydrate or any other organic component, forming a glycoconjugate (Reis *et al.*, 2010; Varki *et al.*, 2009).

Glycoconjugates are involved in many physiological and pathological processes, including the processes of differentiation, cell migration and signaling, host-pathogen interactions and tumor invasion and metastasis (Campbell e Yarema, 2005; Reis *et al.*, 2010).

Glycans classes which are found in eukaryotic cells are defined based on the nature of the glycan binding to the carrier molecule (protein or lipid), forming in this way, various glycoconjugates (fig.4). Thus, glycoconjugates can be classified into proteoglycans, glycosphingolipids, glycosylphosphatidylinositol bound proteins and glycoproteins (Li e Richards, 2010; Varki *et al.*, 2009).

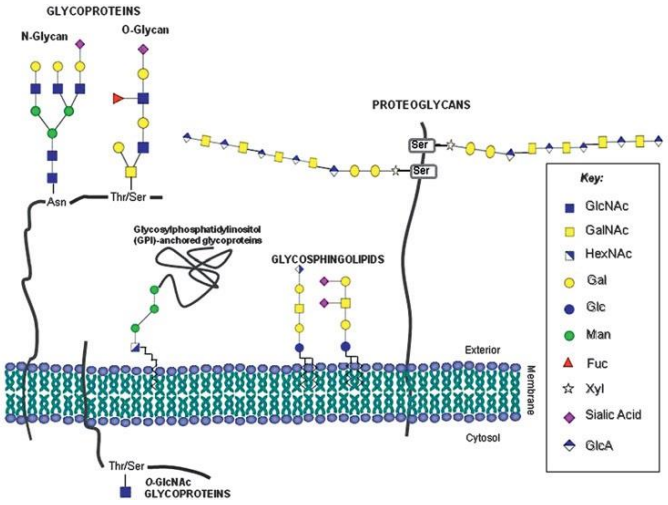


Figure 4 - Glycoconjugates classes expressed in human cells. Adapted from Reis et al., (Reis *et al.*, 2010).

1.7.1) Glycoproteins

Glycoproteins are defined as glycoconjugates in which one or more oligosaccharide chains are covalently linked to a protein (Ohyama, 2008; Reis *et al.*, 2010; Varki *et al.*, 2009). There are two types of glycans associated with glycoproteins based on the type of

attachment: N-glycans and O-glycans. Both types of glycosylation can exist simultaneously within the same molecule and in the same cell. An N-glycan is an oligosaccharide covalently bound to the nitrogen atom of asparagine residues within a type amino acid sequence Asn-X-Ser / Thr (X being any amino acid except proline). O-glycan is a oligosaccharide covalently bound to the oxygen atom of a serine or threonine residue in an amino acid sequence (Li e Richards, 2010; Taniguchi, N., *et al.*, 2008; Varki *et al.*, 2009).

There are many ways in which the O-glycans may be elongated and processed with the addition of terminal residues to these structures, namely the addition of sialic acid, fucose and/or sulfate. These terminal residues often determine the biological function as well as recognition properties of modified glycans (Varki *et al.*, 2009).

1.7.2) Sialic acids

Sialic acids (Sias) constitute a large family of terminal monosaccharides, which include N-acetylneuraminic acid (Neu5Ac) and derivatives thereof, which typically are attached to the expressed glycoconjugates on the cell surface of animal tissues and certain microorganisms (Varki e Schauer, 2009). Sias are involved in many cellular functions, both in physiological and pathological processes, including the regulation of the immune system, triggering infection and progression of certain diseases (Varki, A., Angata, T, 2006; Varki, A., *et al.* 2009) .

Sias are unique sugars that usually occupy the terminal position of the glycan chains and may be modified by external factors, such as pathogens, or upon specific physiological cellular events (sialidases). At cell surface, sialic acid-modified structures form the fundamental key determinants for a number of receptors with known involvement in cellular adhesiveness and cell trafficking, such as the selectins and thesialic acid-binding Ig-like lectins (Siglecs).

Siglecs are the best-characterized I-type lectins involved in the recognition of sialic acids (Angata, T., Brinkman-Van der Linden, 2002) and they are characterized by their

binding specificity for terminal sialic acids (Aarnoudse *et al.*, 2006). Siglecs are expressed predominantly on cells of the immune system (Crocker, Paulson and Varki, 2007), such as human immature DCs and are in particular highly expressed on tolerogenic DCs.

Siglecs are thought to play a role in both positive and negative regulation of immune responses (Crocker, Paulson e Varki, 2007; McMillan e Crocker, 2008) and cells that express high levels of siglecs, such as DCs, are crucial for initiation and differentiation of immune responses (Bax *et al.*, 2011).

1.7.3) Desialylation

Interestingly, sialic acid-modified structures are involved in all DC functions, such as antigen uptake, DC migration, and capacity to prime T cell responses. Sialic acid content changes along DC differentiation and activation and these changes have important implications in DC function.

Videira et al. (Videira *et al.*, 2008) observed that the removal of sialylated structures through treatment with a sialidase diminishes the endocytic capacity of moDCs, suggesting a maturation trigger of these cells. Boog et al. (Boog *et al.*, 1989) showed that removal of sialic acids in non-responder types of APC in mice restores specific failure of T cells to respond to nominal antigen or autoantigen, leading to the idea that sialylation contributes to dampening of immune functions. Crespo et al, (Crespo *et al.*, 2009) indicated that sialidase treatment increases the expression of MHC and of co-stimulatory molecules and affects some functionality of DCs resulting in onset of maturation

1.8) Hypothesis, Aims and Scopes

As reported above sialic acid content affects dendritic cell function and a high sialic acid content is associated with lower efficiency of DC-based immune responses. However,

it is still unclear how sialic acid content affects DC activation and their capacity to prime T cells.

In this thesis, the hypothesis is: the capacity of human dendritic cells to activate antigen specific T cells is most effective when cell surface sialic acids are removed from dendritic cells. We also hypothesized that TLR signaling is improved in desialylated DCs.

The aims of this thesis were:

- To characterize moDCs with altered sialic acid content, by an extrinsic enzymatic treatment with sialidase, evaluating their maturation profile and cytokine expression;
- To analyze the effect of sialic acid shortage in TLR-mediated signaling. To address this we used HEK-cell lines overexpressing Toll-like receptors.
- To investigate effect of sialidase treatment on gp100 peptide (melanoma antigen) binding to MHC-I. For this T2 cells (TAP-deficient) were used.
- To evaluate the ability of dendritic cells with altered sialic acid content to modulate T cell-specific responses.
    - To study $CD8^+$ T-cells activation, we used primary human moDCs treated or untreated with sialidase that were co-cultured with $CD8^+$ T-cell clones, with a specific TCR for the gp100 peptide, in the presence of two types of gp100 peptide: a gp100 short peptide (YLEPGPVTA) and a gp100 long peptide (YLEPGPVTANRQLYPEWTEAQRLDC). The amino acid binding motif for HLA-A*is underlined. HLA-A*0201, an allele of MHC class I, is frequent in the Caucasian population (~50%). Therefore, these epitopes also have the potential to be recognized by gp100 specific $CD8^+$ T cell clones. Peptides derived from the gp100 protein, a known melanoma antigen, were used since there are already several well characterized models available, including T cell clones with specific receptors for peptides derived from this antigen.

- To study $CD4^+$ T cell proliferation, primary human moDCs treated or untreated with sialidase were co-cultured with different ratios of $CD4^+$ T-cells isolated from PBLs.

With this work, we expect to elucidate whether desialylation of human DCs improves the DC capacity to activate T cells, with the potential to improve cytotoxic responses against tumor cells.

2) Materials & Methods

2.1) Human peripheral blood

Human monocytes were isolated from buffy coats of healthy donors (Sanquin, Amsterdam, The Netherlands) through a Ficoll and subsequent Percoll gradient. Informed consent was given by all donors for the use of their blood samples.

2.2) Isolation of monocytes from buffy coats by Ficol and Percol gradient

Peripheral blood mononuclear cells (PBMCs) were isolated by sequential density gradient centrifugation. The first step in this process consists of diluting 50 ml of buffy coat in 130 ml of phosphate buffered saline solution (PBS; BRAUN) containing 1% citrate (PBS-Citrate). This solution was mixed very gently and added to a 50 ml tube containing 10 ml of Ficoll. Afterwards the Ficoll gradient was centrifuged at 700 x g for 30 minutes, without brake.

Ficoll is a hydrophilic polysaccharide with a particular density, superior to PBMCs, but inferior to erythrocytes and granulocytes, allowing a specific separation of the different components, after centrifugation. At this point, blood components are separated by density gradient and four phases are distinguishable: erythrocytes, present as pellet, followed by Ficoll, with the PBMCs at the interface and right beneath the most abundant layer, which corresponds to plasma and the majority of the platelets (fig.5). PBMCs were collected and transferred to 50 ml tubes. PBMCs were washed twice in 50 ml PBS-Citrate. The cell suspension was centrifuged first at

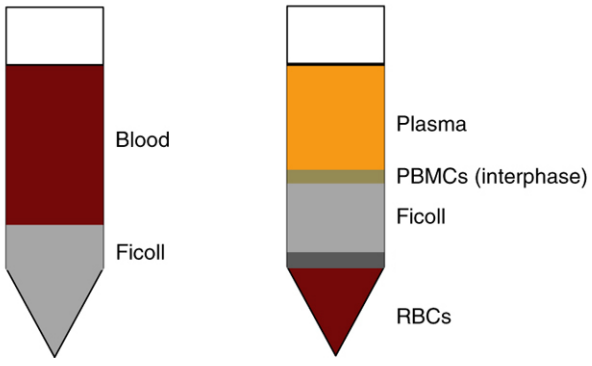


Figure 6 - Layout of separation by ficoll gradient.

400 x g for 10 minutes, and subsequently at 300 x g, for 4 minutes. Afterwards cells were resuspended in 50 ml RPMI-1640 medium (Gibco, UK) supplemented with 10% Fetal Calf Serum (BioWhittaker) and counted using trypan blue (1:10), in order to determine the total number of live PBMCs. Cell count was always performed using the Neubauer chamber (10^4). The total number of live PMBCs is determined as follows:

Total no. live cells = cells counted (without coloration) **× DF × 10^4 × solution final volume** (ml) **(1)**

Before starting with the Percoll isolation the cell were centrifuged once more at 300 x g, for 4 minutes.

The next process was aimed at separating the monocytes and lymphocytes using a Percoll gradient. For this isolation to be successful it is essential to perform the entire process at room temperature (22 °C) because the Percoll is very temperature-sensitive.

PBMCs were resuspended in RPMI-1640 medium supplemented with 10% Fetal Calf Serum (22 °C) at a concentration of 10 million cells per ml. 50 ml tubes were prepared with 15 ml Percoll (Percoll ,1.5M NaCl, MQ water) each and 15 ml of PBMCs were added slowly and carefully on top of the Percoll layer. The Percoll gradient was subsequently centrifuged at 400 x g for 40 minutes, at 22 °C (acc=4, dec=1, added delay 10 minutes). The ring of cells, containing 70% to 96% monocytes depending on the starting amount in PBMC, was collected and transferred to new 50 ml tubes. Tubes were filled up to 50 ml with PBS-citrate, and centrifuged at 400 x g, for 10 minutes, at 22 °C with brake. The cells were washed at least three more times in 50 ml PBS-Citrate and centrifuged at 300 x g, for 4 minutes. Finally, the cells were resuspended in 50 ml RPMI-1640 medium supplemented with 10% Fetal Calf Serum and counted using a trypan blue (1:10), as describe above in **(1)**. The percentage of live "monocytes" from PBMCs is determined as follows:

$$\textbf{\% of live monocytes from PBMCs} = \frac{\textit{total number of monocytes}}{\textit{total number of PBMCs}} \times 100 \quad \textbf{(2)}$$

2.3) Generation of monocyte-derived dendritic cells (moDCs)

After Percoll isolation, the monocytes were resuspended in RPMI-1640 medium supplemented with 10% Fetal Calf Serum (FCS), Interleucine-4 (IL-4, 500 U/ml), and granulocyte macrophage colony-stimulating factor (GM-CSF, 800 U/ml) (both from BioSource/Invitrogen, Carlsbad, CA, USA). 15 million monocytes were seeded per T75 flask in 12 ml medium. The cells were kept in culture at 37 ºC, in a humidified atmosphere with 5% CO_2, to promote the differentiation into dendritic cells (DCs). After 4-5 days the monocytes have differentiated into dendritic cells (moDCs).

2.4) Sialidase treatment

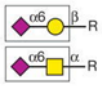
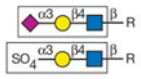
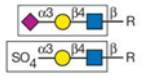
MoDCs (0.5 million of cells) were incubated for 60 min at 37 °C with 25 mU/ml of sialidase (Roche, USA) in serum-free RPMI-1640 medium or DMEM medium (Gibco, UK) and subsequently, washed and resuspended in serum-free RPMI-1640 medium or DMEM medium. In parallel, a control lacking sialidase was incubated under the same conditions containing the same amount of cells.

2.5) Lectin binding assay

The binding of sialic acid-specific plant lectins (Table 1), *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin I and II (MAA II and MAL-I, all from Vector Laboratories, Burlingame, CA) to moDCs with or without sialidase treatment (describe in 2.4 section) was determined by flow cytometry. The cells were incubated with the lectins at a final concentration of 5 µg/ml for 30 minutes at 37°C. Lectins were diluted in Hank's buffered saline solution (HBSS, Gibco, UK) containing 0.5% of BSA (Roche, USA). After washing twice in the same medium the cells were incubated with Alexa 488-labeled Avidine (Molecular probes, USA) for 30 min at 37 °C. After one final washing step

with HBSS/BSA, the samples were transferred to FACS tubes and analyzed in a FACSCalibur (BD Biosciences, San Diego, CA, USA). If the samples could not be measured on the same day, the cells were fixed in 0.5 % of paraformaldehyde in HBSS/BSA.

Table 2 – Lectin specificity. Representation of the structure, the lectin name, the organism origin and its binding specificity. Adapted from Varki, et al.,(Varki *et al.*, 2009).

Structure	Lectin Name	Lectin	organism	Binding specificity
	*Sambucus nigra* aglutinin	SNA	*Sambucus nigra*	Neu5Acα2-6
	*Maackia amurensis* leukoagglutinin	MAA II	*Maackia amurensis*	Neu5Ac/Gcα2-3 O-glycans
	*Maackia amurensis* leukoagglutinin	MAL I	*Maackia amurensis*	Neu5Ac/Gcα2-3 N-glycans

2.6) MoDC stimulation

After sialidase treatment of the moDCs, as describe in 2.4 section, treated and untreated moDCs were plated ($1x10^5$ cells/well) and incubated overnight, at 37 °C in the presence or absence of Toll-like Receptor (TLR) stimulation.

The TLR ligands (Table 1) used were: Lipopolysaccharide (LPS (Escherichia coli) - TLR4-ligand, 10 ng/ml; Sigma-Aldrich), Triacylated lipopeptide (Pam3CysK4 - TLR2-ligand, 5 µg/ml; Invivogen), or Resiquimod (R848 - TLR7/8-ligand, 5 µg/ml; Invivogen). TLR ligands were diluted in RPMI-1640 supplemented with 1000 U/ml penicillin/streptomycin (Lonza), 2 mM glutamine (Lonza) and 10% FCS (BioWhittaker) – RPMI-1640 complete medium.

After O/N incubation, moDCs were centrifuged at 300 x g for 2 minutes and 65 µl of supernatant was harvested and frozen at -80 °C for future evaluation of cytokine secretion by ELISA. Subsequently, cells were resuspended in 100 µl of PBA (PBS -Fisher Scientific, USA, with 0.5 % of BSA and Azide) and transferred to a V-bottom plate, whereby each original well was divided into two separate wells in the V-bottom plate. After washing at 300 x g for 3 minutes, cells were incubated for 30 minutes at 4 °C with phycoerythrin-conjugated antibodies to CD markers (BD Biosciences, diluted in PBA) for the analysis of DC maturation. The CD markers that we used were CD83, CD80 and CD86 (all from BD Biosciences). After incubation, cells were washed with 100 µl PBA, centrifuged at 300 x g for 3 min and then resuspended in 100 µl of PBA and transferred to FACS tubes. Cells were analyzed on the FACS Calibur.

2.7) Toll-Like Receptor (TLR) test

The Human Embryonic Kidney (HEK) cells (kindly provided by Douglas Golenbock, University of Massachusetts Medical School, Worcester, USA) is a genetically modified cell line that expresses TLR-2 (HEK-TLR2), TLR-4 (HEK-TLR4), or TLR-8 (HEK-TLR8). They can be used to determine TLR activation upon the stimulation with respective TLR ligand by assessing IL-8 production (only cytokine secreted by these type of cells). These cell lines were grown in DMEM medium supplemented with 1000 U/ml penicillin/streptomycin, 10 % FBS and 0.5 mg/ml geneticin (G418). 2.5 million of cells were treated or left untreated, as described in 2.4 section.. After washing, cells were plated ($1x10^5$ cells/well in 100 µl of DMEM medium supplemented with 1000 U/ml penicillin/streptomycin and 10% FBS) in a 96 wells flat bottom plate and allowed to adhere for 90 min. Afterwards 50 µl of medium was removed and 50 µl of TLR ligand were added in different concentrations: LPS (Escherichia coli, TLR4-ligand, 100-1 ng/ml; Sigma-Aldrich), Pam3CysK4 (TLR2-ligand, 5-0.5 µg/ml; Invitrogen) or R848 (TLR8-ligand, 50-0.5 µg/ml; Invitrogen). Cells were incubated for 24 hours at 37 ºC in a humidified

atmosphere with 5% CO_2. After incubation 50 µl of supernatant was harvested for IL-8 analysis by sandwich ELISA.

2.8) Enzyme-linked Immunosorbent Assay (ELISA)

The cytokine levels in supernatants (IL-10, IL-12p70, IL-8, IL-6, tumor necrosis factor alpha (TNF-α), interferon-γ (INF-γ)) were determined by Sandwich ELISA using an antibody pairs from Life Technologies. The capture antibody was resuspended in coating buffer (50 mM Na_2CO_3, pH 9.7) (APPENDIX I) and added to the 96 well ELISA plate (100 µl/well) overnight (ON) at 4 ºC. After two washes with 200 µl/well ELISA washing buffer (PBS 1x containing 0.05% Tween20 (Sigma Aldrich)) wells were blocked with 200 µl/well of blocking buffer (PBS 1x and 1% BSA) for 30 minutes at 37 ºC. After two more washes, 100 µl of samples were added, in duplicate, to each well. Depending on the type of cytokine measured, the sample dilution was adjusted (Table 4). Human cytokine Standards were diluted in blocking buffer (APPENDIX I) to make a standard curve. In addition, 50 µl of the detection antibody diluted in blocking buffer (APPENDIX I) was added to each well. Plates were sealed and incubated for 2 hours at room temperature (RT) under mild shaking. After washing 4 times with ELISA washing buffer, 100 µl/well of streptavidin-HPR (Invitrogen, 1:10.000 dilution in ELISA washing buffer) was added and incubated at RT for 30 minutes. After extensive washing with ELISA washing buffer (6 times) 100 µl/well of substrate solution (100 µl of 10 mg/ml 3,3′,5,5′-tetramethylbenzidine (TMB), 1 µl 30% H_2O_2 in 10 ml of substrate buffer (0.1 M citric acid/0.1 M sodium acetate, pH 4.0)). The reaction was stopped by the addition of 50 µl of 0.8 M H_2SO_4. Plate were read at 450 nm and analyzed by software microplate manager 5.2.1.

Table 3 - Sample dilutions to cytokine measured by sandwich-ELISA.

Cytokine	Dilution
IL-6	1:100
IL-8	TLR-2 : 1:10
IL-8	TLR-4 : 1:2
IL-8	TLR-8 : 1:2
IL-10	1:20
IL-12p70	1:20
TNF-α	1:200
INF-γ	1:3

2.9) Real-Time PCR (qPCR)

For determining the mRNA expression levels of interleukin (IL)-6, IL-10, IL-12p40 and TNF-α) in moDCs a qPCR was used. MoDCs were treated prior with sialidase or left untreated (shown in 2.4 section) and after over-night incubation with TLR stimulation (shown in 2.6 section) cells were lysed and mRNA was isolated at indicated time-points using an mRNA Capture kit (Roche, Indianapolis, IN, USA). In short, lysates were incubated with biotin-labeled oligo$(dT)_{20}$ for 5 min at 37°C and subsequently transferred to streptavidin-coated tubes and incubated for 5 min at 37°C.

After washing twice, cDNA was synthesized using the Reverse Transcription System kit (Promega, Madison, WI, USA) following manufacturer's guidelines. In brief, 30 µl of the reverse transcription mix was added (5 mM $MgCl_2$, 1× reverse transcription buffer, 1 mM of deoxyribonucleotides phosphated (dNTP), 0.4 U recombinant RNasin ribonuclease inhibitor, 0.4 U of avian myeloblastosis virus reverse transcriptase (AMV RT), 0.5 µg random hexamers in nuclease-free water) and incubated for 10 min at room temperature, 90 min at 42°C, and 5 min denaturing at 99°C.

Then the amplification was performed with specific primers for each cytokine referred to above. Primers were designed using Primer Express 2.0 (Applied Biosystems, Carlsbad, CA, USA) and synthesized by Invitrogen. Glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) was used as an endogenous reference gene (García-Vallejo *et al.*, 2004).

The reaction mixture consisted of 4 µl Fast SYBR Green Master Mix (Applied Biosystems), 0.2 µl of the primer solution containing 5 nmol/µl of both primers, 1.8 µl H_2O, and 2 µl of a 1:2.5 dilution of the cDNA solution. PCR reactions were run for 2 min at 50°C, followed by 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min 60°C. PCR reactions were run on a ViiA 7 sequence detection system (Applied Biosystems). The Ct value is defined as the number of PCR cycles where the fluorescence signal exceeds the threshold value which is fixed at 10 times the standard deviation of the fluorescence during the first 15 cycles and typically corresponds to 0.2 relative fluorescence units (Vliet, Van *et al.*, 2013). GAPDH served as an endogenous reference gene (García-Vallejo *et al.*, 2004).

The Ct values normalized for target and GAPDH mRNA (Nt) is determined as follow:

$$\mathbf{Nt} = 2^{Ct(GAPDH) - Ct(target)} \quad \mathbf{(3)}$$

2.10) DC loading

Tumor antigen used for DC loading are usually a peptide derived from a tumor associated protein. In this assay, the tumor antigen is a peptide derived from the gp100 protein, a known melanoma antigen. Peptides were synthesized and purity was determined as described previously (Hooijberg *et al.*, 2000). The peptide was selected for binding to specific major histocompatibility complex (MHC) molecule, thus guarantying that it will be presented to peptide-specific T cells as described previously (Fehres *et al.*, 2015; Skipper *et al.*, 1999).

Two types of gp100 peptide were used: a gp100 short peptide (YLEPGPVTA) and a long gp100 long peptide (YLEPGPVTANRQLYPEWTEAQRLDC). The amino acid sequence underlined binds to HLA-A*0201, an allele of MHC class I, that is frequent in the

Caucasian population. The gp100 long peptide is internalized through phagocytosis and during processing the peptide is cleaved and the resultant short peptide (underlined) becomes available to form a peptide–MHC-I complex and to be presented through MHC class I to $CD8^+$ cytotoxic T cells, a phenomenon known as antigen cross-presentation. In this way, these epitopes have the potential to be recognized by gp100 specific $CD8^+$ T cell clones.

2.11) Peptide binding assay

For this assay a T2 - TAP (Transporter Associated with Antigen Processing) deficient cell line was used.

TAP-competent cells translocate endogenously generated peptides into the endoplasmic reticulum (ER) where they are loaded onto MHC class I molecules. These peptides are derived from cellular proteins degraded in the proteasomal complex (Kisselev *et al.*, 1999). TAP-deficient cells lack the transporter for MHC class I-restricted peptides to enter the ER endoplasmatic reticulum. The absence of ER-localized peptides in TAP-deficient cells results in the accumulation of empty MHC class I molecules in the ER-Golgi intermediate compartments as well as on the cell surface. These empty MHC molecules can be rapidly loaded by available peptides (Raposo *et al.*, 1995). Therefore, exogenous peptides can potentially be loaded onto MHC complexes at the cell surface directly.

First of all the T2 cells were cultured till confluency and then harvested (4×10^6cells/mL). The cells were divided and washed. Half of the cells (2×10^6cells/mL) were incubated with sialidase and the other half (2×10^6cells/mL) was incubated without sialidase as described in section 2.4. After washing the cells were resuspended in serum-free RPMI-1640 medium with 6 µg/ml of β_2-microglobulin (Sigma-Aldrich, St. Louis, MO) and plated 100.000 cells/well (50 µl/well).Then, $gp100_{280–288}$ (short) peptide (YLEPGPVTA) was used in three different concentrations: 100 µM, 10 µM and 1 µM. After 4 hours of incubation at 37 °C the cells were transferred to a V-bottom plate and

thereafter the T2 cells were washed with PBA (PBS with 0.5% of BSA and Azide) and stained with PE-labelled anti-HLA-A2$^+$ antibody (clone BB7.2, PharMigen) for 30 min, at 4 °C. Lastly, the cells were washed with PBA and transferred to tubes to be analysed. Peptide-mediated stabilization of HLA-A2$^+$ molecules at the cell surface of T2 cells was detected by flow cytometry. Figure 2 is a schematic representation of T2 binding assay.

For each assay, lectin binding assay was performed in parallel to verify the efficiency of sialidase treatment, as previously described in section 2.4.

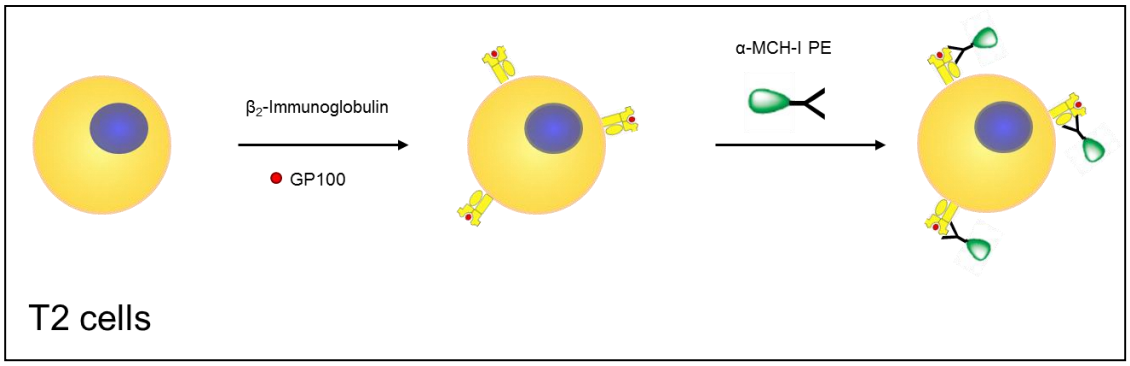


Figure 7 - Summary of the MHC-I binding assay with T2 cells. Prior to the assay T2 cells were treated with sialidase or left untreated. After a wash step T2 cells were resuspended in serum-free RPMI-1640 medium with β2-microglobulin and plated in the presence of gp100$_{280–288}$ (short) peptide. After 4 hours of incubation at 37 °C the cells were stained with PE-labelled anti-HLA-A2$^+$ antibody.

2.12) Activation of CD8$^+$ T cell clones

MoDCs were treated with sialidase (5 × 10^6 moDCs) or left untreated (5 × 10^6 moDCs) as described in section 2.4, and then were washed. After this, LPS (100 ng/ml; 25 µl/well) was added or not to half of the treated cells and half of the untreated cells and then the cells were plated (30,000 cells/well; 25 µL/well). The peptides were added (50 µL/well) in different concentrations: gp100 long peptide (30 µM, 10 µM, 3 µM, and 1 µM); gp100 short peptide at 10 µM. The short peptide is used as a positive control, because it can immediately be loaded onto MHC-I molecules displayed at moDCs cell surface and thus easily be presented to T cells, without being requiring further processing.

After 3 h of incubation at 37 °C the moDCs were washed twice with serum-free RPMI-1640 medium, before they were co-cultured with the $CD8^+$ T cells. The $CD8^+$ T cells was originated from a $CD8^+$ T clone with a TCR specific for the gp100 peptide (as described in 2.10 section) were thawed and washed two times with 25 ml of in RPMI-1640 complete medium, then resuspended in IMDM medium (Gibco, UK) and added 100.000 cells/well (100 µl/well) to 96-well-plate. T cells (100.000 cells/well) with (10 ng/ml) of phorbol myristate acetate (PMA) and 500 ng/ml of ionomycin (Io) (both from Sigma) are used as a positive control for the assay (graph not shown).

After overnight or 3 days of incubation at 37 °C, supernatants were harvested and IFN-γ levels were measured by sandwich ELISA using specific antibody pairs from Biosource as described in section 2.8.

2.13) $CD4^+$ T cell Proliferation

MoDCs were treated with sialidase (5×10^6 moDCs) or left untreated (5×10^6 moDCs) as described in section 2.4, and then washed. After this, LPS was added or not, as described in previous section and the cells were incubated for 3 h at 37 °C.

$CD4^+$ T cells were prepared as follows: Peripheral blood lymphocytes (PBLs) HLA-$A2^+$ (obtained by Ficol/Percol isolation from peripheral blood) were thawed and then washed three times with serum-free RPMI medium. Autologous $CD4^+$ T cells were isolated using a MACS $CD4^+$ T cell negative isolation kit (Miltenyi Biotec), according to the manufacturer's rules. In short, PBLs were washed with 25 ml of buffer (PBS + 2mM of EDTA + 1% FCS) and after centrifugion, 400 µl of MACS buffer and 100 µl Ab-cocktail (a cocktail of biotin-conjugated antibodies against CD8a, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC-class II, Ter-119 and TCRγ/δ as primary labeling reagent) were added to PBLs (100 x 10^6 cells) and incubated 10 min at 4ºC. Then 200 µL of beads were added to PBLs and incubated for 15 min at 4ºC. After this, the cells were washed with 25 ml of MACS buffer and the isolation was done. Finally $CD4^+$ T cells

were washed, resuspended in IMDM medium and distributed in wells (100.000 cells/well) in a volume of 50 µl.

After the incubation with LPS, the moDCs were washed with serum-free RPMI-1640 medium and added at different ratios: 1:5; 1:10; 1:25; 1:50; 1:100 to $CD4^+$ T cells. In order to detect proliferation of the $CD4^+$ T cells, after 3 days of co-culture [^{3}H]-thymidine incorporation into DNA (1 µCi/well; Amersham Biosciences, NJ, USA) was measured. After 24 h, cells were harvested onto filters and [^{3}H]-thymidine incorporation was assessed using a beta scintillation counter.

2.14) Statistical analysis

Results were analyzed using a Student's t-test using GraphPad Prism 6 software (GraphPad Software, San Diego, CA). Results were considered to be significantly different when $p<0.05$.

3) Results

This work had as a primary goal to evaluate how the desialylation of DCs affected their functionality, in particular their capacity to present antigen and activate T cells. To address this question, we first had to establish a method to desialylate DCs, based on the extrinsic treatment of DCs with a bacterial sialidase.

3.1) Sialidase protocol optimization

In order to analyze the effects of sialidase treatment on moDCs, the sialidase protocol was optimized first. To evaluate this, lectin binding studies with SNA (α2-6-linked sialic acids), MAA II (α2-3-linked sialic acids on *O*-glycan) and MAL I (α2-3-linked sialic acids on *N*-glycan) were done, to confirm that immature moDC express high levels of α-2,3- and α-2,6-sialic acids (Videira *et al.*, 2008). Different concentration of sialidase (20 mU/ml and 40 mU/ml) and different incubation times (1h and 2h) were tested (fig.7).

At both concentrations tested, sialidase can cleave α2-3-linked sialic acids on O- and N-glycans from the moDCs surface more efficiently than α-2.6 sialic acids, as shown by the large decrease in MAA II and MAL I binding. SNA binding does not seem to be affected by this treatment. (fig.7, a1)). However, there are no significant differences between the two concentrations used (20 mU and 40 mU).

In the graph of % of positive cells, we can see that sialidase treatment can only affect the percentage of positive cells that bind to MAA II and this decrease is even bigger when 40mU/ml of enzyme was used, however 20 mU/ml also shows a visible decrease (fig.7, a2)).

Incubation times of 60 min and 120 min are both effective, since at both time points a decrease in binding to all lectins can be observed (fig.7, b1)). In this graph (fig.7, b1)) SNA binding was reduced by the treatment with 20 mU/ml of sialidase, not observed in fig.7, a1, but the results derived from different donors witch can be influence.

Again treatment only affected the % of MAA II positive cells (fig.7, b2)), which agrees with the outcome above shown (Fig.7, a2)).

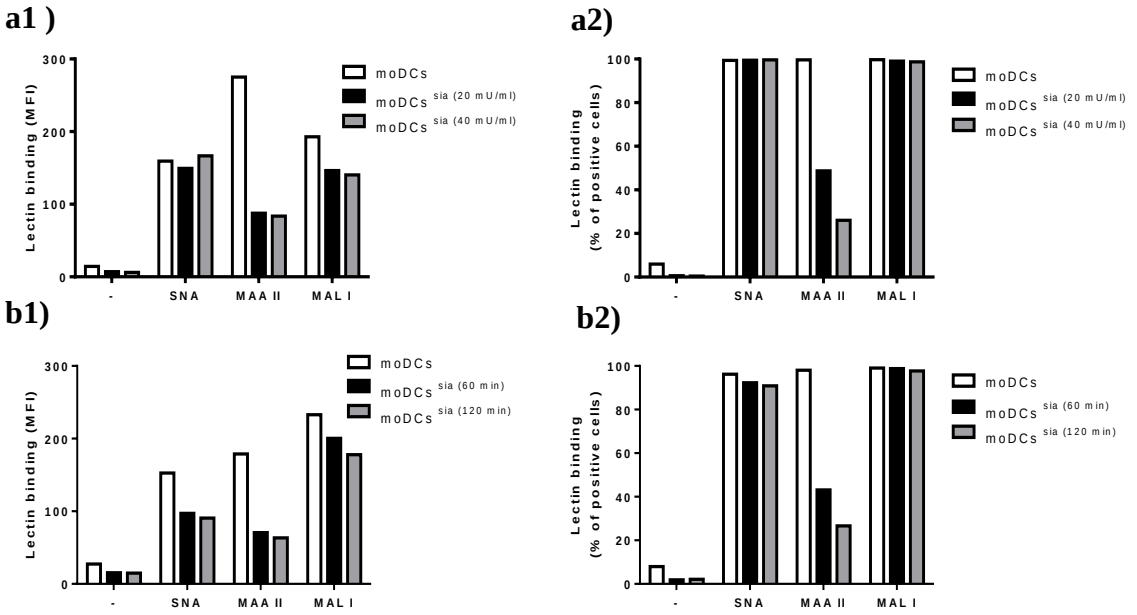


Figure 12 - Lectin binding to cell surface of moDCs. MoDCs were stained with Sambucus nigra lectin (SNA - recognizing α2,6-sialic-acids) and Maackia amurensis lectin (MAA II - recognizing α2,3-sialic-acids on O-glycans and MAL I - recognizing α2,3-sialic-acids on N-glycans) following sialidase treatment. **a)** Immature mo-DCs were treated with sialidase at two different concentrations (20 mU/ml and 40 mU/ml) or left untreated, for 60 min at 37 °C (n=1). Control results are represented in white, 20 mU of sialidase in black and 40 mU in grey: **a1)** Mean fluorescence intensity (MFI); **a2)** the percentage of positive cells. **b)** Immature mo-DCs were treated with 20 mU/ml of sialidase at two different incubation times (60 min and 120 min) or left untreated for 60 min at 37 °C (n=1). Controls results are represented in white, 60 min of incubation in black and 120 min of incubation in grey: **b1)** Mean fluorescence intensity (MFI) ; **b2)** the percentage of positive cells. The results were obtained by flow cytometry, the results displayed in a) and b) are derived from different donors.

Given these results, we decided to proceed with a standard treatment protocol using 25 mU/ml of sialidase for 60 min at 37°C. The final concentration of sialidase chosen was 25 mU/ml and not 20 mU/ml only for practical reasons in the experimental set up.

3.2) Removal of sialic acid from the moDC surface by sialidase treatment increased co-stimulatory molecule expression when stimulated with TLR-ligands

Previous studies (Cabral *et al.*, 2013; Crespo *et al.*, 2009; Videira *et al.*, 2008) have shown that altering the surface desialylation on moDCs by sialidase treatment has several functional consequences. The triggering of maturation in moDCs by sialidase treatment can be an explanation for this observation, because maturation causes structural and functional alterations in moDCs.

To assess the maturation status of the moDCs after sialidase treatment, we analyzed, by flow cytometry, the expression of maturation markers CD80, CD83 and CD86. Effects of Sialidase treatment were evaluated with or without stimulation with TLR-ligands. TLR-ligands are generally used as inducers of maturation, as can be observed by the increased expression of co-stimulatory molecules in TLR-stimulated cells (fig.8)

However, an upregulation in co-stimulatory molecules expression can be seen in sialidase treated moDCs, especially in moDCs stimulated with R848 (TLR7/8 ligand), where a significant increase in all maturation markers was observed (fig.8). After stimulation with LPS, a significant increase in CD80 and CD86 markers was also observed in treated cells. The CD83 marker is upregulated in cells treated with the Pam3 stimulus.

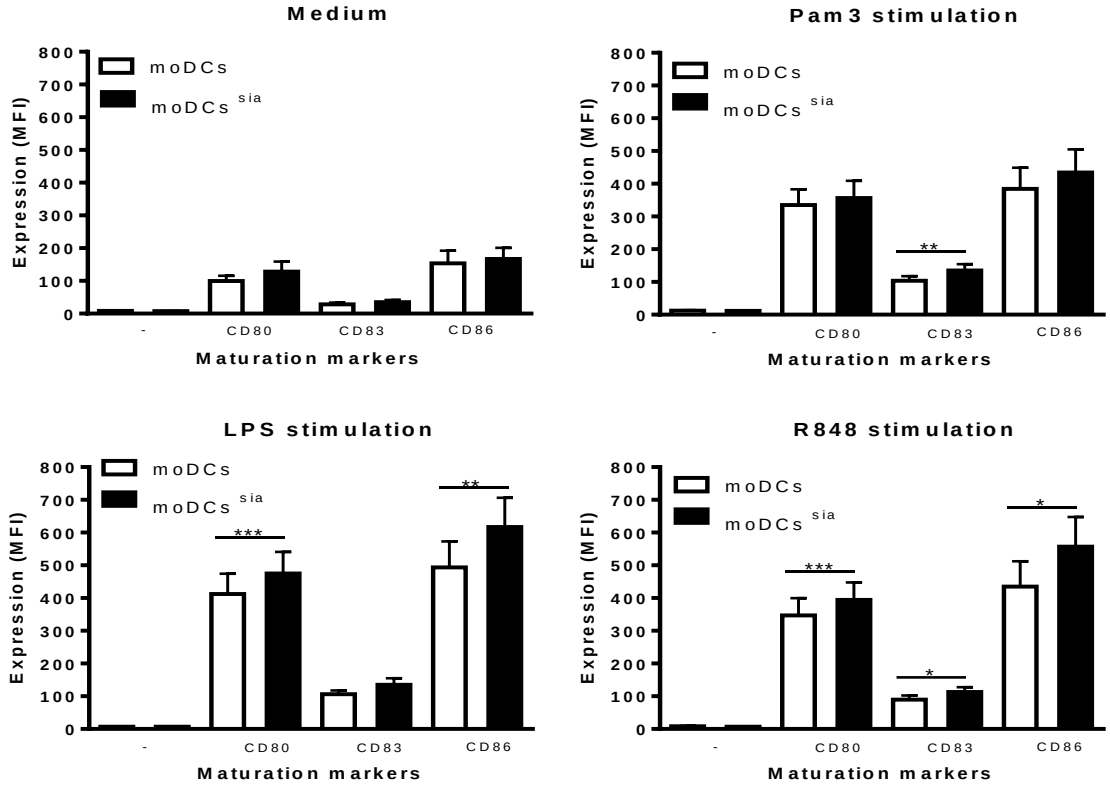


Figure 13 - Expression of maturation markers, CD80, CD83 and CD86, in moDCs after stimulation with Pam3, LPS and R848 (TLR-2, TLR-4 and TLR-7/8 ligands, respectively). moDCs were treated with sialidase (60 min at 37 °C) or left untreated. After overnight incubation with TLR-ligands, moDCs were stained with antibodies to different CD markers. Co-stimulatory molecule expression was evaluated by flow cytometry. The results are the average mean fluorescence intensity (MFI) of 15 independent assays. Statistical significance (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$) refers to the difference between untreated (white bar) and sialidase-treated moDCs (black-bar).

To evaluate the efficiency of the sialidase treatment to remove sialic acids on moDCs surface, a lectin binding assay was carried out in parallel. As mentioned previously, during monocyte differentiation into moDCs, an increase in sialylated structures, specifically, α-2,3 and α-2,6 sialic acids is observed, showing the highest expression at day 4 (Videira *et al.*, 2008).

In this assay the moDCs after the 4th day of differentiation were labeled with SNA (α2-6-linked sialic acid), MAA II (α2-3-linked sialic acid on O-glycans) and MAL I (α2-3-

linked sialic acid on N -glycans) after sialidase treatment and the MFI was measured by flow cytometry. (fig.9).

Adramatic decrease in MFI of MAA II and MAL I to moDCs treated with sialidase can be observed. The % of positive cells for MAA II binding MAA II decreased to approximately 40% after sialidase treatment.

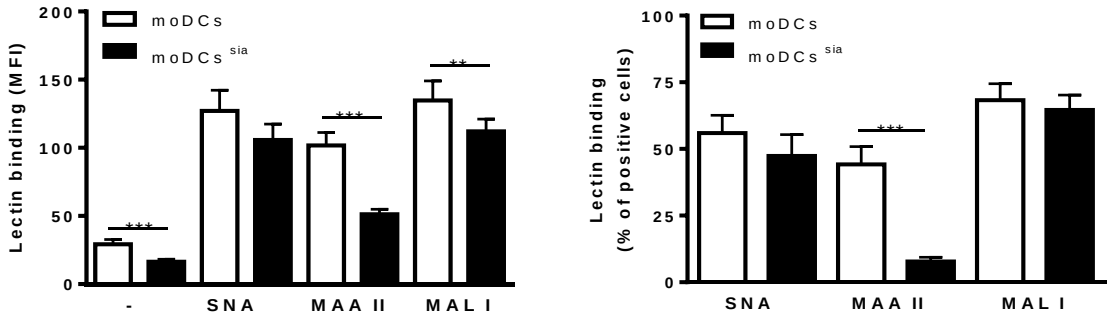


Figure 14 - Plant lectin binding assay. moDCs (day4 after of differentiation) were treated with sialidase (60 min at 37 °C) or left untreated and then were labelled with the different plant lectins (SNA, MAA II and MAL I). lectin binding was evaluated by flow cytometry. The results are the average mean fluorescence intensity (MFI) (left side) or percentage of positive cells (right side) of 16 independent assays. Statistical significance (**$p \leq 0.01$; ***$p \leq 0.001$) refers to the difference between untreated (white bar) and sialidase-treated moDCs (black bar).

3.3) Cytokine secretion is not affected in moDCs treated with sialidase

To complement the results obtained by flow cytometry, where the sialidase treatment increased the expression of co-stimulatory molecules, suggesting the induction of maturation in moDCs, we analysed the secretion of several cytokines, IL-10, IL-12p70, IL-6 and TNF-α by ELISA, after over-night stimulation with different TLR-ligands (fig.10).

Our results suggest that sialidase treatment does not significantly affect the production of cytokines by moDCs in any of the conditions tested. However, it is possible to see a tendency for an increase of cytokine secretion by treated-moDCs in the presence of LPS and a tendency for the decrease of cytokine secretion by -moDCs in the presence of the R848 stimulus, especially for IL-10, IL-6 and TNF-α (fig.10).

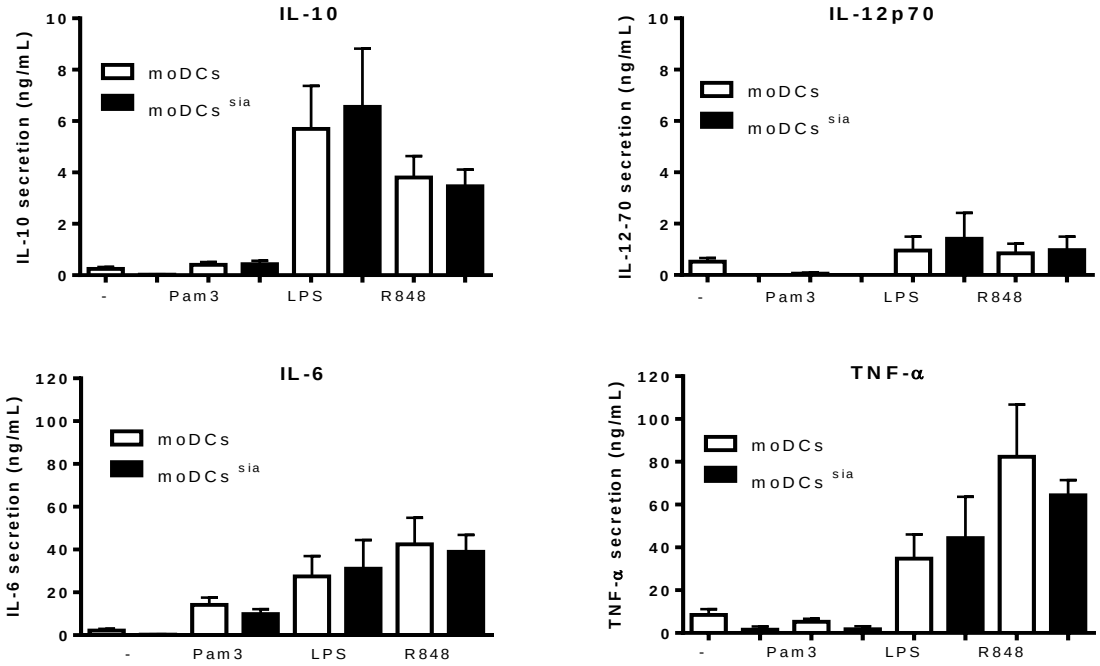


Figure 15 - Cytokine secretion. MoDCs were treated with sialidase (black bar) for 60 min at 37 °C or left untreated (white bar). After over-night incubation with different TLR-ligands the supernatant was harvested and cytokine production was measured by ELISA. The results are the mean ± SEM of 9 independent experiments.

Previous studies (Cabral *et al.*, 2013; Crespo *et al.*, 2009) , where cytokine levels wereanalyzed by qPCR-real time, did show an increase in expression in treated-moDCs compared to untreated moDCs.

As the results of the present study didn't support the literature, we decided to analyze cytokine production of one donor, by ELISA and qPCR-real time, in order to compare the results obtained by both techniques (cytokines secretion and transcript levels of cytokines, respectively). All cytokines studied before (IL-6, IL-10, IL-12p70 and TNF-α) in treated and untreated moDCs were assessed by ELISA. IL-6 and TNF-α were measured after 6h of incubation after ON incubation. As IL-10 and IL-12p70 could probably not be detected after 6 h, they were only measured after ON incubation. All incubations were done in the presence of TLR-ligands (fig.11).

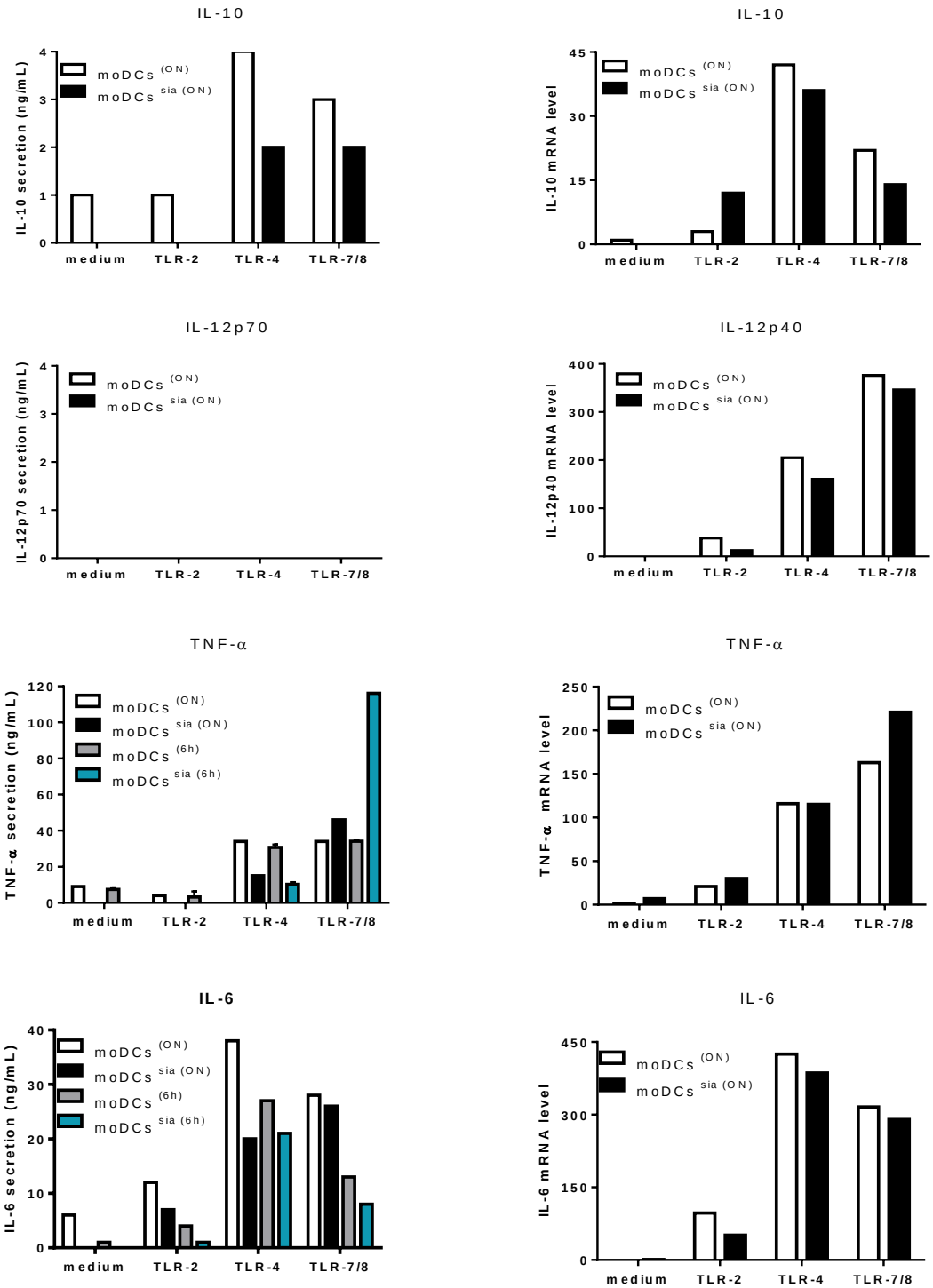


Figure 16 - Cytokine production analysed by ELISA (left side) and qPCR (right side). moDCs were treated with sialidase (60 min at 37 °C) or left untreated. After incubation for 6 h (grey bar and blue bar) or over-night (white bar and black bar) with different TLR-ligands, the supernatant was harvested and the cytokine production was measured by ELISA. Cell pellets were lysed and cytokines mRNA levels were measured by qPCR. The results are of 1 independent experiment.

With the exception of IL-12p70 that was not detectable by ELISA, for the other cytokines it was possible to compare results obtained by both techniques. The secretion of IL-10, IL-6 and IL-12p70 seem to be decreased in cells that have previously suffered the sialidase treatment, while TNF-α secretion was found to increase in the presence of R848 stimulus. Overnight incubation led to higher cytokine concentrations compared to only 6 h incubation (fig.11)

Thus, our results still do not match the literature, but we can see that for the same donor, transcript levels of cytokines and the extracellular secretion followed the same pattern, which leads us to conclude that the analysis by ELISA is reliable. .

3.4) Sialidase treatment and TLRs activation

In order to elucidate the mechanism of action of sialidase we proceeded to test whether we could observe a direct effect of sialidase treatment on TLR activation. To test this, HEK cell lines stably expressing specific TLR receptors were used (HEK-TLR-2, TLR-4 and TLR-8),.

The treatment with sialidase in HEK cell lines was done according to the treatment used for moDCs, as described in section 2.4. each cell line (HEK-TLR-2, TLR-4 and TLR-8) was subsequently incubated ON at 37 °C with the respective stimulus (Pam3, LPS, or R848). After incubation, supernatant was harvested to measure the IL-8 cytokine secretion by ELISA (fig.12).

As shown in fig 12, sialidase treatment does not affect IL-8 production in either the TLR-4 nor TLR-8 receptors (fig.12). On the other hand, TLR-2 activation appears to be affected by sialidase treatment, since there was a little decrease in IL-8 production, although not significant, in treated cells, when the Pam3-ligand was used as a stimulus (fig.12)

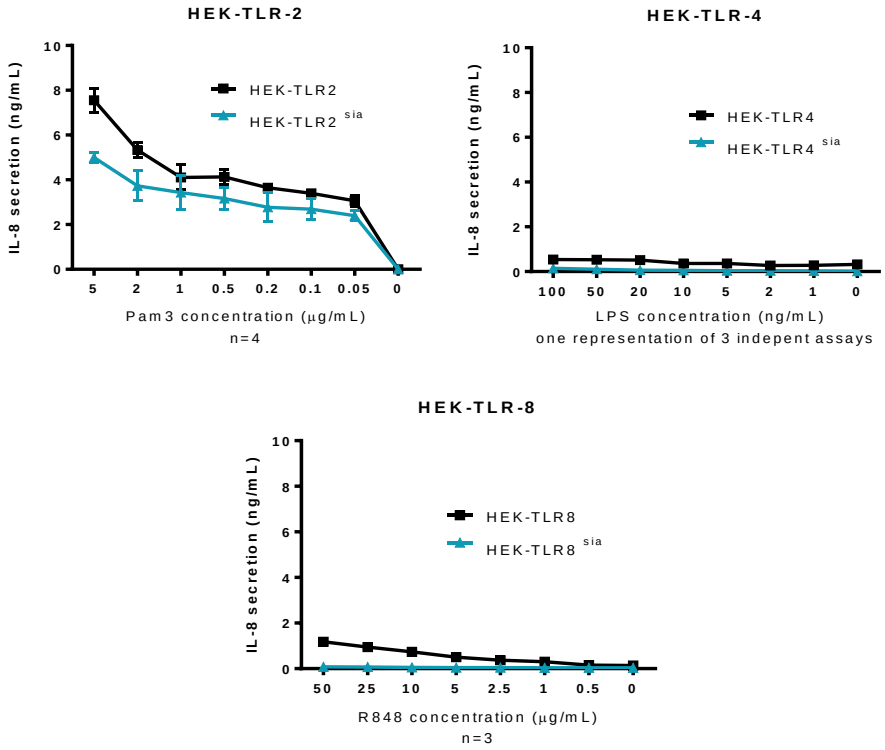


Figure 17 - Toll like receptor test. HEK cells were treated with sialidase for 60 min at 37 °C or left untreated. After incubation over-night with different TLR-ligands the supernatant was harvested and the IL-8 production was measured by ELISA. TLR-2 results are the mean ± SEM of 4 independent experiments, TLR-4 results are of one representation experiment out of 3 and the TLR-8 results are the mean ± SEM of 3 independent experiments. Statistical significance (*$p \leq 0.05$) refers to the difference between untreated (black line) and sialidase-treated (blue line) HEK cells.

3.5) Desialylation effect in gp100 short peptide binding to HLA-A2 in T2 cell line

To understand whether treatment with sialidase had any effect on the binding of gp100 peptide to MHC-I, a binding assay with a T2 cell line was carried out.

This cell line is TAP deficient, implying that only when peptide binds to MHC-I, it remains on the cell surface, whereas otherwise the MHC-I is endocytosed and recycled. For this test, cells were treated for 1h at 37 °C with sialidase or left untreated, and then washed. The cells were subsequently incubated 3/4h or ON with different concentrations of gp100 short peptide (1 µM, 10 µM and 100 µM) in the presence of β-microglobulin to help the stabilization of the peptide-MHC I-complex. Thereafter, cells were stained with a specific

antibody to anti-HLA-A2 and analyzed by flow cytometry (fig.9, a)). In parallel, the cells were also labelled with lectins (SNA, MAA II and MAL I) to check the efficiency of sialidase treatment (fig.9, b)).

The lectin binding results demonstrate that sialidase treatment was effective, as shown by the more than 50% decrease in lectin binding to the surface of the T2 cells (fig.9, b)).

The peptide binding results are not significant, but we can still observe a little increase in the levels of MHC-I in treated cells when they are incubated with peptide but also without peptide (fig.9, a)). Treated and untreated cells have a very parallel stabilization curve. Moreover, T2 cells treated with sialidase but without peptide have also higher levels of MHC-I which may indicate that, besides the stabilization effect, it seems that MHC-I is higher expressed on the surface than T2 cells treated with sialidase (fig.13, a)).

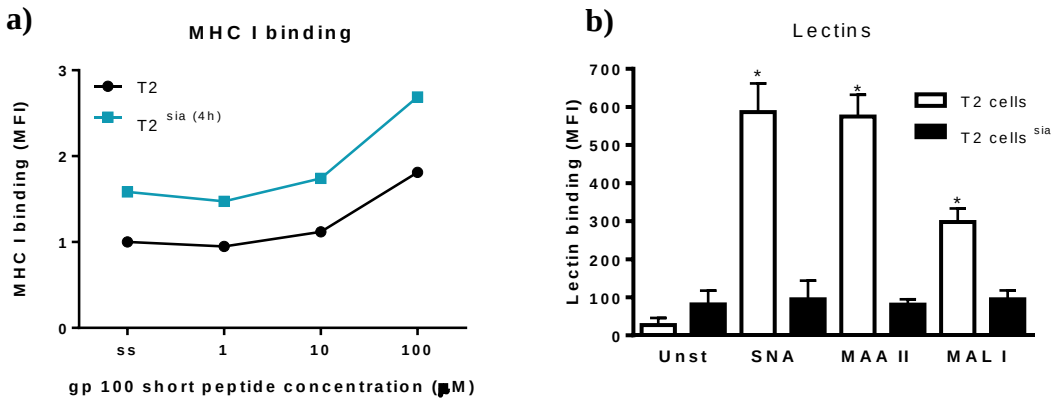


Figure 18 – gp100 peptide bound MHC-I **is more stable** on sialidase treated T2 cells. **a)** T2 binding assays were performed by incubating clls, treated (blue line) or not (black bar) with sialidase, without peptide (ss why not just call this zero?) or with different concentrations of gp100 short peptide in the presence of β-microglobulin for 4h at 37 ºC. After incubation, T2 cells were washed and stained with anti- HLA-A*02 antibody, for 30 min at 4 ºC and analyzed by flow cytometry. Results were normalized to the condition without peptide and without sialidase treatment. Graph show the mean ±SEM of at least 4 independent experiments. **b)** As a control T2 cells were treated (black bar) with sialidase or left untreated (white bar) and the cells were labeled with different plant-lectins (SNA, MAA II and MAL I) to ensure the effectiveness of sialidase treatment. Graph shows the mean ±SEM of at least 4 independent experiments and statistical significance (*$p \leq 0.05$) refers to the difference between untreated and sialidase-treated T2 cells.

3.6) Sialidase treatment of moDCs improves their ability to activate tumor antigen gp100-specific T cells

DCs have a unique ability to present exogenous antigens via MHC-I molecules, a function designated as cross-presentation. Exogenous antigens (gp100 long, in this case) are degraded by proteasomes and coupled to MHC-I molecules for presentation to naïve $CD8^+$ T lymphocytes (CTL). This process ensures that DCs can create a cytotoxic immune response against tumor or infected cells. The CTL are characterized by the production of IFN- γ and TNF- α cytokines.

To assess if the activation of gp100 tumor antigen -specific $CD8^+$ T cell clones was enhanced by moDCs treated with sialidase the IFN- γ production by these T-cells was measured, in a presence of two types of peptide, gp100 short and gp100 long peptide (described in 2.10 section).

The gp100 long peptide is internalized through phagocytosis and during processing the peptide is cleaved and the resultant short peptide (underlined in 2.10 section) becomes available to form a peptide–MHC-I complex and to be presented through MHC class I to $CD8^+$ cytotoxic T cells, a phenomenon known as antigen cross-presentation. In this manner, the epitopes have the potential to be recognized by gp100 specific $CD8^+$ T cell clones.

The usual treatment of moDCs with sialidase was done, as describe in section 2.4, LPS was added (fig.14, b) and d)) or not (fig.14, a) and c)) to treated or untreated cells and then the cells were plated. The peptides were added in different concentrations: gp100 long peptide: 30 μM, 10 μM, 3 μM, 1 μM; gp100 short peptide: 10 μM. After 3 h of incubation at 37 °C cells were washed and the $CD8^+$ T cell clones specific for gp-100 peptide were added. After ON (fig.14, a) and b)) or 3 day (fig.14, c) and d)) incubation at 37 °C, supernatants were harvested and IFN-γ levels were measured by sandwich ELISA. Results were normalized to the IFN-γ levels obtained with the incubation with short peptide without sialidase treatment.

As shown in fig.14, a), sialidase treatment of moDCs without without LPS stimulation appears to increase their ability to activate $CD8^+$ T cell clone after overnight incubation. This observation is more prominent in the presence of gp100 long peptide at concentrations between 30-10 µM. With lower peptide concentrations, sialidase treatment did not alter the activation of T cells. Furthermore, the activation of T cells co-cultured with moDCs treated with sialidase is also increased in the presence of gp100 short peptide. That is, both the direct presentation and the cross-presentation appear to be more effective in moDCs treated with sialidase.

However, when $CD8^+$ T cells were kept in culture with moDCs for 3 days (fig.10, c) and d)), the difference in activation of T cells by moDCs treated and untreated appears to be greater when moDCs are stimulated with LPS specially in the presence of 30-3 µM of gp100 long peptide and gp100 short -peptide.

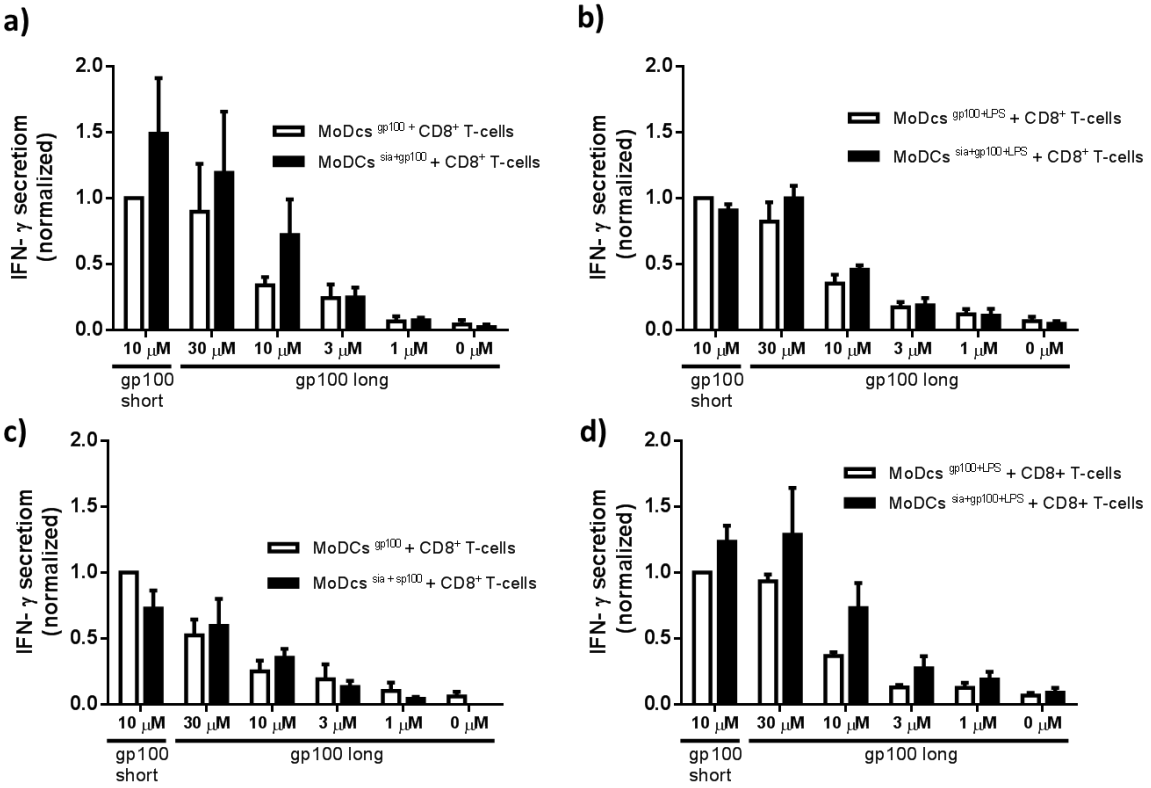


Figure 19 - Improved production of IFNy by $CD8^+$ T cell clones. **a)** gp100-specific $CD8^+$ T cells were co-cultured overnight with gp100-loaded moDCs, that have previously been treated (black bar) or not (white bar). **b)** gp100-specific $CD8^+$ T cellswere co-cultured overnight with gp100-loaded moDCs and LPS, after treatment (black bar) or not (white bar) with sialidase. **c)** gp100-specific $CD8^+$ T cellswere co-cultured for 3 days with gp100-loaded moDCs after treatment (black bar) or not (white bar) with sialidase. **d)** gp100-specific $CD8^+$ T cells were co-cultured for 3 days with gp100-loaded moDCs and LPS after treatment (black bar) or not (white bar) with sialidase. Different concentrations of long gp100 peptide, which is cross-presented to $CD8^+$ T cells, were used. The short gp100 peptide was used as a positive control for the functionality and antigen specificity of $CD8^+$ T cell clone. The secretion of the IFN-γ cytokine was measured by ELISA (n=3). Results were normalized to the IFN-γ levels after incubation with short peptide without sialidase treatment (a) and c)) or with short peptide without sialidase treatment and with LPS (b) and d)).

3.7) Sialidase treatment of moDCs improves proliferation of autologous $CD4^+$ T cells

The proliferation of autologous $CD4^+$ T cells was assessed by [^{3}H]-thymidine assay. The usual treatment with sialidase was done to moDCs, as describe in section 2.4LPS was added or not (Fig.9, a) and b) respectively) to treated or untreated cells for 3 h at 37 °C. Autologous $CD4^+$ T cells were isolated and co-cultured with moDCs in different ratios: 1:5;

1:10; 1:25; 1:50; 1:100. After 3 days (at 37°C) [^{3}H]-thymidine was added for 24 h to detect incorporation into the DNA of proliferating CD4$^+$ T cells.

Figure 9 shows that compared to untreated moDCs the moDCs treated with sialidase, irrespective of their LPS stimulation, seem to increasee CD4$^+$ T cell proliferation. T cells display a maximal proliferation at the ratio is 1:25 when co-cultured with immature moDCs (Fig.9, **a)**). At a ratio of 1:50 there is a significant increase in the proliferation of T cells co-cultured with treated moDCs (Fig.9, **a)**). The proliferation of T cells in the presence of mature moDCs is maximal at a ratio of 1:10 (Fig.9, **b)**). At a ratio of 1:100 a significant increase is observed in the proliferation of T-cells co-cultured with moDCs treated with sialidase (Fig.9, **b)**).

To obtain significant results it will be necessary to repeat the assay to increase the sample size.

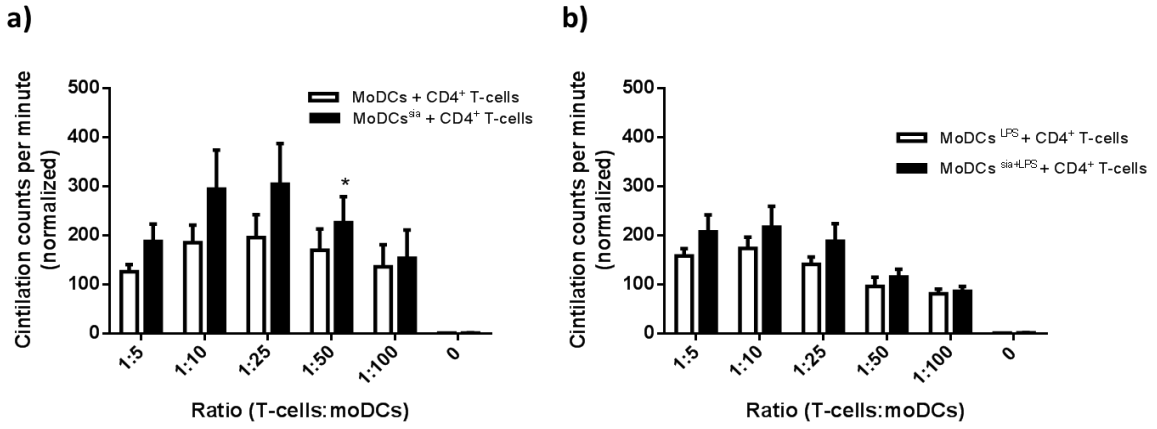


Figure 20 - Sialidase treatment of moDCs improves proliferation of autologous CD4$^+$ T cells**.** The proliferation of purified CD4$^+$ T cells was measured by [^{3}H]thymidine uptake assay, after a challenge with different ratios of moDCs that were treated (black bar) or not treated (white bar) with sialidase, in the absence a) or in the presence b) of LPS for 3 days (n=3). Results were normalized to the condition without moDCs and without sialidase treatment (a) or without moDCs, without sialidase treatment and with LPS (b). Graphs show the mean ± SEM of at least 3 independent experiments**.**

4) Discussion and conclusions

With the progression of the glycobiology field, sialic acids have revealed an important role in the modulation of immune responses and are therefore a therapeutic target of great interest. However, our knowledge is still not as thorough to the point where we can understand all the mechanisms involved in its action.

It has been reported by many authors that the α2-6 and α2-3 sialylated structures on the surface of some immune cells are modulated according to cell differentiation and maturation (Marino *et al.*, 2004; Moody *et al.*, 2003).

Dendritic cells in particular, are antigen presenting cells bearing a large endocytic capacity (Cabral *et al.*, 2013). It has been demonstrated by several researchers that human monocyte-derived dendritic cells (moDCs) are highly sialylated (Sallusto *et al.*, 1995; Videira *et al.*, 2008). These sialylated structures modulate the operation of moDCs, for instance their endocytosis capacity, degree of activation and maturation (Crespo *et al.*, 2009; Silva, Konstantopoulos e Videira, 2012), which are all key features in the regulation of immune response mediated by lymphocytes. Due to its potent immunoregulatory capacity, dendritic cells are very promising therapeutic targets in the use for vaccines for cancer therapy.

It is now well accepted that the content of sialic acid on the cell surface can be modulated by endogenous sialidases such as Neu1 (Kooyk, van *et al.*, 2004) as well as by sialidases from exogenous sources, for instance, released by pathogens in the course of an infection (Cabral *et al.*, 2013).

This thesis is aimed at evaluating the structural/functional consequences of elimination of sialic acids on the moDCs surface through the action of an exogenous sialidase, a hydrolase which cleaves the glycosidic bonds of neuraminic acids (α2-3, α2-6 and α2-8).

First we show that treatment with sialidase and subsequent addition of Toll like receptors (TLR) agonists induces dendritic cell maturation, thereby increasing the amount of some costimulatory molecules on the surface of these cells especially when stimulated with R848 (fig.8). However, in the absence of any stimulus, sialidase treatment alone does not interfere with the maturation moDCs (fig.8), suggesting that the maturation effect is the result of both triggers simultaneously. *Amith et al.* (Amith *et al.*, 2010), describe that in primary BM macrophage cells, the removal of α-2,3-linked sialyl residues by Neu1 sialidase is essential for TLR dimerization and subsequent cellular signaling to activate NFκB. It is possible that the action of the sialidase results in increased responsiveness of the moDCs to the TLR agonists (LPS, PAM3, R848). As described by *Amith et al.* (Amith *et al.*, 2010) TLR4 receptors have less α-2,3-sialylated structures after activation by LPS when compared to the non-activated TLR4.

Furthermore, during the desialylation has cross intermediary molecules signaling between the cell surface and TLRs as immunoregulatory Siglecs (Crocker, Paulson e Varki, 2007; O'Neill, 2008). Human moDCs express several receptors, such as Siglecs, that recognize sialic-acid-containing glycans, that may have preferences for either the Sia(α2,3) linked to Gal(β1,3)GalNAc or the Sia(α2,6) to Gal(β1,4)GalNAc (Varki e Angata, 2006). Siglecs have the potential to interact not only in trans with sialylated ligands found in other cells, but also in cis with ligands found at the surface of the same cell (Varki e Angata, 2006).

When Siglecs bind to sialic acid containing glycans, they trigger an intracellular inhibitory signal which prevents the signaling of membrane TLRs as no production of inflammatory cytokines is found (Stamatos *et al.*, 2010). However, in the presence of sialidase, these sialic acids are removed and there is a reduction in binding to Siglecs, abrogating the inhibitory signaling therefore downstream events in the signaling pathway of TLR continue, culminating in inflammatory cytokine production (Paulson e Kawasaki, 2011).

When moDCs were treated with the sialidase enzyme and stimulated with the TLR7/8 ligand R848, a significant increase was observed in all co-stimulatory molecules studied, CD80, CD84 and CD86 (fig.8). As the TLR7 and 8 receptors are intracellular, it will be necessary to perform sialidase internalization studies to identify the signaling pathway responsible for this increase.

While the enzyme treatment has led to extensive desialylation, as measured by significant reduction in the binding of lectins MAA II and MAL I to moDCs (fig.9), there were no significant differences in the detected amount of cytokines in the medium of treated cells with sialidase and in the medium of untreated cells (fig.9). This was not expected since the moDCs showed signs of maturation (fig.8), which could result in the production of inflammatory cytokines.

It is possible that desialylation of glycoconjugates is important for intracellular cytokine production and the amount of sialidase was not sufficient to activate the production of these effectively. Another reason relates to the possibility that the sialidase action is reversed by regeneration of sialic acids to the cell surface by means of sialyltransferase activity present in or on the DCs, as described by S*tamatos et al.* (Stamatos *et al.*, 2010), and therefore does not modulate the most delayed mechanism involved in cytokine production.

Earlier, our laboratory already verified (Cabral *et al.*, 2013) that an increase in cytokine production in moDCs treated with sialidase. However, the enhancement in cytokine production by moDCs treated with sialidase occurred only in some of the donors (fig.10) whereas in other donors cytokine production in treated moDCs production was lower than in untreated moDCs or even sometimes no differences were found. These results have not been reproducible, and all together, show no significant differences in cytokine production from sialidase treated and untreated moDCs.

Thus, it was necessary to quantify the production of cytokines by ELISA (evaluating the overall amount of cytokines produced by moDCs into the medium), and by Real Time-PCR simultaneously, (assessing the amount of mRNA corresponding to each

intracellular cytokine). Besides Real Time-PCR is a more sensitive technique that was also used by *Cabral et al.* (Cabral *et al.*, 2013). In our study the amount of cytokines showed a slight increase in moDCs treated with sialidase as measured by both techniques (fig.11). However, the results still did not show significant differences so it would be necessary to increase the sample size to draw conclusions.

By comparing the present results with the previous results by Cabral *et al*. (Cabral *et al.*, 2013) it is also necessary to take into account the difference in the method used for the isolation of monocytes. The isolation method used here was a Ficoll/Percol gradient, whereas *Cabral et al.* (Cabral *et al.*, 2013) was used isolation by positive selection of $CD14^{+}$ monocytes (MACS). As several authors describe (Elkord *et al.*, 2005; Normann *et al.*, 2010; Zhou *et al.*, 2012) the isolation method of monocytes can influence the activation state of the dendritic cells and this could modulate the cytokine production.

In order to elucidate the mechanisms of sialidase treatment on TLR triggering, sialidase assays were carried out in HEK-TLR transfectants. The results of these experiments suggest that sialidase treatment did not affect TLR triggering as no significant differences were found in the production of the IL-8 cytokine (fig.12). This result was unexpected since in dendritic cells the desialylation seems to involve in TLR mechanism (fig.8), but this activation may be a consequence of the action of the sialidase on Siglecs as described above. This leds us to consider that the two different cell types (moDCs versus HEK-TLR transfectants) might respond differently to desialylation, thus providing a possible reason for these results, although it is interesting to study the mechanism with other cellular models.

Crespo et al. (Crespo *et al.*, 2009) showed that MHC expression increases in sialidase treated moDCs, and therefore we analyzed whether desialylation directly affects the stability of MHC-I in a T2 binding assay. MHC class I antigen presentation and cell surface expression depends primarily on peptide transport into the ER/Golgi by the transporter for antigen presentation (TAP) (Lankat-Buttgereit e Tampe, 2002).

T2 cells are deficient in TAP but still express low amounts of MHC class I on the cell surface (*No Title*, [s.d.]). The T2 binding assay is based upon the ability of gp100 short peptide (YLEPGPVTA) to stabilize the MHC class I complex on the surface of the T2 cells. Although no significant differences were observed between treated and untreated cells (fig.13, a)), a slight increase in the direct binding of gp100 short peptide, especially in the presence of concentrations of peptide 10-100 µM, to MHC-I cells treated with the enzyme was observed. However, even in sialidase treated cells that were incubated without peptide (fig.13, a)) a slight increase in the expression MHC-I could be seen,which leads us to consider that, regardless of the presence of peptide, the treatment with the enzyme alone can affect this complex and therefore its basal expression. This is even supported by extensive desialylation as shown by a significant decrease in lectins binding (SNA, MAA II and MAL I) to treated T2 cells (fig.13, b)).

Together, these results show that the action of sialidase might directly interfere with peptide binding to MHC-I, and subsequently the presentation to T cells, but to conclude this, it would be necessary to repeat the test to a reasonable sample size.

In addition to the direct presentation of peptide to MHC-I, the ability of DCs to cross-present tumor antigens has long been a focus of interest to scientists that aim to establish efficient cell-based cancer immune therapy. cross-presentation allows exogenous antigens to access the processing and presentation machinery of a cell so that exogenous antigenic peptides are displayed on MHC class I molecules for T cell recognition, which consequently leads to the priming of $CD8^+$ T cell responses (Fehres *et al.*, 2014). As such, the cross-presentation pathway is essential for inducing cytotoxic T-lymphocyte (CTL) responses against tumor antigens (Platzer, Stout e Fiebiger, 2014). Indeed, cross-priming of tumor reactive cytotoxic $CD8^+$ T cells through cell-based tumor vaccines is a major goal in cancer immunotherapy (Andersen e Ohlfest, 2012; Apetoh *et al.*, 2011). Hence, it is important to better understand the mechanisms that underlie the induction of tumor-specific cytotoxic T-lymphocyte (CTL) responses initiated by DCs via cross-presentation.

to mimic this process *in vitro,* the capacity of sialidase treated and not treated moDCs , in the presence (Fig. 14, b) and d)) or absence (Fig.14, a) and c)) of LPS, to activate $CD8^+$ T cell clones was assessed. We used different concentrations of two gp100 peptides, the gp100 short peptide that is presented directly to MHC I and consequently can used as a positive control and the gp100 long peptide that needs to be cleaved intracellularly to be cross-presented via MHC I.

A $CD8^+$ T cells was used that contains a TCR that specifically recognizes part of amino acid sequence of the gp100 protein (YLEPGPVTA). MoDCs were cultured overnight (fig.14, a) and b)) or 3 days (fig.14, c) and d)) with $CD8^+$ T cell clone and the amount of IFN-γ released by T-cells was measured by ELISA.

The results of the fig.8 show that, the amount of gp100 peptide, sialidase treatment, stimulation with LPS and the culture time are all factors that appear to determine the activation of the cytotoxic T cells. In the absence of LPS the activation of the $CD8^+$ T cell clone appears to be upregulated when they are cultured overnight with sialidase treated moDCs, in the presence of both gp100 short peptide and gp100 long peptide at concentrations of 30-10 μM (Fig. 14, a)). Thus, both the direct presentation as well as the cross-presentation of the long gp100 peptide seems to be improved under these conditions, since the production of IFN-γ increases, so the effect on sialidase may affect both pathways. When cytotoxic T cells were incubated with moDCs without LPS after 3 days their activation (fig.14, b)) in general is smaller, as there was no difference when using sialidase treated moDCs or untreated moDCs. So to compare the consequence of sialidase effect on the moDC-mediated activation of $CD8^+$ T cells it is better to co-culture both cells ON.

However, the opposite happens when T cells are incubated with mature moDCs. In treated and LPS-stimulated moDCs overnight incubation with the $CD8^+$ T cell clone did not increase the activation of these T cells both in the presence of gp100 short and gp100 long peptides (fig.14 b)), but if the incubation is continued for at least 3 days, the activation of T cells appears to increase under these conditions (fig.14, d)), which leads us to consider

that the activation of $CD8^+$ T cells by treated moDCs in the presence of LPS requires at least 3 day of incubation before the highest activation of these T cells can be noted.

It would be very important to increase the number of assays in these tests, since these were not enough to get statistically significant results, but it is noticeable that the sialidase treatment has a tendency to enhance the activation of $CD8^+$ T cells.

To further investigate the functional effect of sialidase treatment on moDCs, we also evaluated the ability of either sialidase-treated or untreated moDC to induce proliferation of autologous $CD4^+$ T cells. A defining aspect of the adaptive immune system is its capacity to elicit antigen-specific cellular immune responses by the instruction of antigen-specific $CD4^+$ and $CD8^+$ T cells. This property is entirely based on the presentation of antigen in MHC molecules (the peptide–MHC complex) and its recognition by the T cell receptor. The loading of extracellular antigen in MHC-II, recognized by $CD4^+$ T cells, occurs in a different intracellular compartment than the loading of antigen in MHC-I, recognized by $CD8^+$ T cells (Fehres *et al.*, 2014).

Autologous $CD4^+$ T cells were isolated from peripheral blood using a MACS $CD4^+$ T cell negative isolation kit and co-cultured with sialidase-treated or untreated moDCs, with or without LPS stimulus, in different ratios: 1:5; 1:10; 1:25; 1:50; 1:100. After 3 days, proliferation of the CD4+ T cells was assessed by [^{3}H]-thymidine incorporation.

According to our results, the number of proliferating $CD4^+$ T lymphocytes is significantly increased when moDCs without LPS stimulation lose their surface sialic acids (fig.15, a), especially in a ratio of 1:50.

This study did not evaluate the expression of MHC in sialidase-treated moDCs, however, our laboratory has previously demonstrated that sialidase-treated human DCs have increased expression of major histocompatibility complex (MHC) and costimulatory molecules, increased gene expression of specific cytokines and induce a higher proliferative response of T lymphocytes (Crespo *et al.*, 2009). Together, these data suggest that

clearance of cell surface sialic acids contributes to the development of a T helper type 1 pro-inflammatory response.

In conclusion, an increased sialidase activity in DCs may be capable of enhancing the immunogenicity of antigens and that removal of sialic acid unmasks the hidden epitopes.

Sialidase treatment in DCs would have several advantages over the currently used DCs maturation protocols. Sialidase treatment together with TLR stimuli (LPS, PAM3, R848, for example) increase the maturation status as evidenced by the increased expression of costimulatory molecules on the moDCs surface. Moreover, these results demonstrate that sialidase treatment in moDCs seems to promote proliferation of helper T cells via presentation in MHC class II but also activation of cytotoxic T cells via direct presentation and cross-presentation of antigenic gp100 peptides in MHC class I. This means that both $CD4^+$ and $CD8^+$ tumor-specific T cells could be induced in patients by vaccines based on DCs with altered sialylation.

The action of sialidases is not clear yet, but in the model with TAP-deficient T2 cells the MHC I seem to be affected by the sialidase treatment. As such, it would be important to continue these studies to obtain a better understanding of the relationship between sialidase and MHC I.

The model with the HEK-cells did not confirm a direct action of sialidase in activation of the TLR, but it would be good to test other model systems, seen that one of the results of sialidase treatment may indirectly affect these receptors. In future trials we should replicate the test with other cell types, that are structurally similar (in terms of surface receptors) to moDCs, since HEK-cells are a simplistic model in this term.

Further functional studies are still required to understand the competence and immune modulatory capacities of desialylated DCs. Nevertheless, because surface sialylation influences the immunogenicity of DCs upon antigen loading, the findings of this thesis should have a particular relevance to DC-based therapies and they propose that

sialylation is an issue to be considered when refining the immunological parameters of vaccination with DCs.

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7) Appendixes

Appendix 1 - Human Cytokine sandwich ELISA protocol

Material:

- Capture antibodies

Cytokine	Manufacturer	Catalogue Number	Concentration stock	Coating	Dilution
IL-1ß	Biosource	AHC0612	1.0 mg/ml	1 µg/ml	1/1000
IL-4	Biosource	AHC0642	1.0 mg/ml	1 µg/ml	1/1000
IL-6	Biosource	AHC0562	1.0 mg/ml	0.5 µg/ml	1/2000
IL-8	Biosource	AHC0982	1.0 mg/ml	0.5 µg/ml	1/2000
IL-10	eBioscience	14-7108-85	0.5 mg/ml	0.25 µg/ml	1/2000
IL-12p40	Biosource	AHC8122	1.0 mg/ml	1 µg/ml	1/1000
IL-12p70	eBioscience	14-7128-82	0.5 mg/ml	0.5 µg/ml	1/1000
TNFα	Biosource	AHC3712	1.0 mg/ml	1 µg/ml	1/1000
IL-17A	eBioscience	14-7178-85	0.5 mg/ml	0.5 µg/ml	1/1000
IFNγ	Biosource	AHC4432	1.0 mg/ml	1 µg/ml	1/1000

- Detection antibody

Cytokine	Manufacturer	Catalogue Number	Concentration stock	Detection	Dilution
IL-1ß	Biosource	AHC0519	0.5 mg/ml	0.75 µg/ml	1/700
IL-4	Biosource	AHC0749	0.5 mg/ml	0.4 µg/ml	1/1250
IL-6	Biosource	AHC0469	0.5 mg/ml	0.2 µg/ml	1/2500
IL-8	Biosource	AHC0789	0.5 mg/ml	0.2 µg/ml	1/2500
IL-10	eBioscience	13-7109-85	0.5 mg/ml	0.25 µg/ml	1/2000
IL-12p40	Biosource	AHC7129	0.5 mg/ml	0.2 µg/ml	1/2500
IL-12(p40/p70)	eBioscience	13-7129-81	0.5 mg/ml	0.5 µg/ml	1/1000
TNFα	Biosource	AHC3419	0.5 mg/ml	0.5 µg/ml	1/1000
IL-17A	eBioscience	13-7179-85	0.5 mg/ml	0.5 µg/ml	1/1000
IFNγ	Biosource	AHC4539	0.5 mg/ml	0.5 µg/ml	1/1000

- BSA - Bovine Serum Albumine Fraction V, Roche 1075094001, stored at 4°C
- PBS 10x - Fisher Bioreagents BP399-1

- Assay Diluent (or Blocking buffer) - Dissolve 0.5 gram of BSA in 100 ml of PBS 1x.
- Cytokine standards - stock of 10 ng/ml rec cytokine is stored at -20ºC, this is generated from a lyophilized stock (stored at 4ºC). Dilute powder by adding assay diluent to a concentration of 10 ng/ml. Incubate 15 minutes RT and aliquot 125µl/epje.
  NB IL-10 standard is stored at -80ºC, freezer 2: **2.G.2** For IL-12p70 ELISA, use IL-12p40 standard.
- Streptavidin-HRP - Biosource, SNN2004, aliquoted and stored at -20ºC.
  Working aliqoute is stored at 4ºC.
- Coating buffer - 50mM Na_2CO_3 buffer pH 9.7.
  Dissolve 2.65 g of Na_2CO_3 (Merck 6398.5000) in 490 ml of Milli-Q. Adjust to pH 9.7 and fill up to 500 ml. Buffer is stored at 4ºC
- 25% Tween20 solution - Dissolve 10ml of Tween20 (Sigma P5927) in 30ml PBS
- ELISA wash buffer - Add 2ml of 25% Tween20 and 100ml of PBS 10x to 898 ml of Milli-Q
- Blocking buffer - Dissolve 1 gram of BSA in 100 ml of PBS 1x.
- DMSO - Fluka, 41647
- 10mg/ml TMB - Dissolve 50mg of TMB (Sigma T2885) in 5 ml of DMSO
- Substrate-buffer - Dissolve 21.02 g of citric acid (Merck 244, $C_6H_8O_7 \cdot H_2O$) and 8.2 g of sodium acetate (J.T Baker, 0258, CH_3COONa) in 990 ml of Milli-Q and adjust to pH 4.0 with acetic acid (Riedel de Haen, 33209). Fill up to 1 liter
- Substrate solution - Add 100 µl of 10 mg/ml TMB and 1 µl 30% H_2O_2 in 10 ml substrate-buffer. Use immediately.
- Stop Solution - 0.8 M H_2SO_4: Add 21 ml pure H_2SO_4 (Sigma 32,050-1) to 479 ml of Milli-Q
- Maxisorp 96 well ELISA plate - NUNC 442404
- ELISA reader - Bio-Rad Benchmark

Protocol:

1. Coat NUNC maxisorp 96 well ELISA plate with 100µl/well coating antibody (concentration and dilutions: see above) in coating buffer
2. Seal the plate and incubate overnight at 4ºC.
3. Discard wells and wash two times with 200µl/well ELISA wash buffer.
4. Block wells with 200µl/well of blocking buffer.
5. Incubate the plate 30 minutes at 37ºC
6. Discard wells and wash 2x with 200µl/well (ELISA wash buffer)
7. Add 100µl/well sample/standard, diluted in assay diluent. Use standards in duplicate!

IL-10 standard:

1:	conc= 2000 pg/ml	5 ul stock	+ 495 ul PBS/BSA
2:	conc = 800 pg/ml	200 µl from 1	+ 300 µl PBS/BSA
3:	conc = 320 pg/ml	200 µl from 2	+ 300 µl PBS/BSA
4:	conc = 128 pg/ml	200 µl from 3	+ 300 µl PBS/BSA
5:	conc = 52 pg/ml	200 µl from 4	+ 300 µl PBS/BSA
6:	conc = 20 pg/ml	200 µl from 5	+ 300 µl PBS/BSA
7:	conc = 8 pg/ml	200 µl from 6	+ 300 µl PBS/BSA
8:	blank		300 µl PBS/BSA

Standard all other cytokine dilutions: (stock-solution, 10 ng/ml)

1:	conc = 2000 pg/ml	100 µl stock	+ 400 µl PBS/BSA
2:	conc = 800 pg/ml	200 µl from 1	+ 300 µl PBS/BSA
3:	conc = 320 pg/ml	200 µl from 2	+ 300 µl PBS/BSA
4:	conc = 128 pg/ml	200 µl from 3	+ 300 µl PBS/BSA
5:	conc = 52 pg/ml	200 µl from 4	+ 300 µl PBS/BSA
6:	conc = 20 pg/ml	200 µl from 5	+ 300 µl PBS/BSA
7:	conc = 8 pg/ml	200 µl from 6	+ 300 µl PBS/BSA
8:	blank		300 µl PBS/BSA

8. After pipetting standards/samples, add 50µl/well of detection antibody (concentration and dilution: see above) in assay diluent
9. Seal plate and incubate at RT for 2 hours, shaking 100 rpm.
10. Discard wells and wash 4 times with 200µl/well ELISA wash buffer.
11. Add 100µl/well of 1:10.000 diluted streptavidin-HRP in ELISA wash buffer
12. Seal plate and incubate at RT for 30 minutes
13. Discard wells and wash 6 times with 200µl/well ELISA wash buffer.
14. Add 100µl/well of substrate solution to each well
15. Incubate plate at RT for approximately 15 minutes (maximal 30 minutes!)
16. Add 50µl of stop solution to each well
17. Using ELISA reader, read plate at 450nm and analyse data by software microplate manager 5.2.1
18. Settings in analysis for standard curve report:

Regression type:	Cubic
Transformation:	Log (conc)-linear (abs)
Axis Transformation:	Log(x)-linear(x)